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(71) Applicant: UNIVERSITY TECHNOLOGIES INTERNATIONAL INC. [CA/CA]; Suite 204, 609-14 Street, N.W., Calgary, Alberta T2N 2A1 (CA).

(72) Inventor: SANTAMARIA, Pere; 812 72nd Avenue, N.W., Calgary, Alberta T2K 0P6 (CA).

(74) Agent: MITCHELL, Richard, J.; Marks & Clerk, P.O. Box 957, Station B, Ottawa, Ontario K1P 5S7 (CA).

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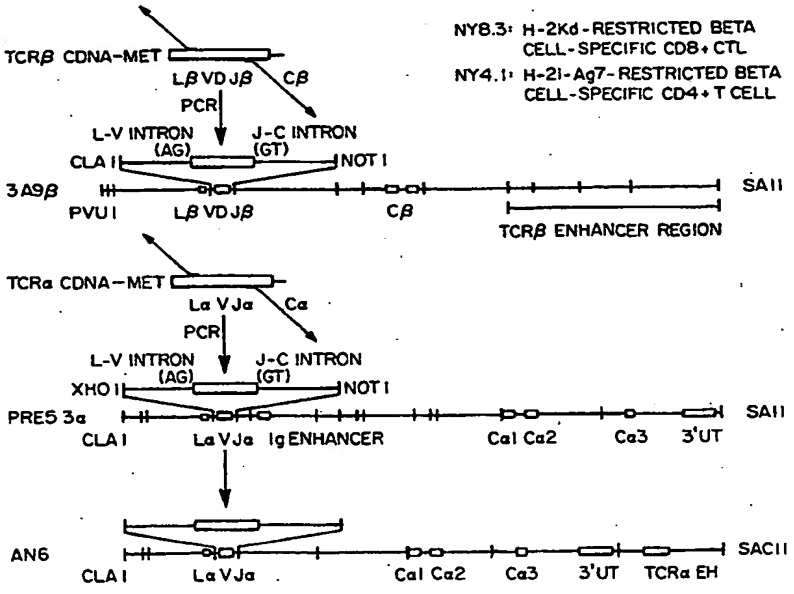

(57) Abstract

This invention relates generally to transgenic animal models useful in testing agents for inhibition of CD4 + and/or CD8+T-cell destruction of pancreatic beta cells. In particular, it relates to transgenic mice which have T-cell receptor α and/or β chain genes derived from beta cell cytotoxic CD4 + or CD8 + T cells in their genome. The present invention also relates to methods of making T-cell-receptor transgenic animals developing early-onset diabetes, and to methods of evaluating agents for the prevention and treatment of insulin-dependent *diabetes mellitus*.

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TRANSGENIC ANIMALS EXPRESSING DIABETOGENIC T-CELL RECEPTOR TRANSGENES

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Provisional Application Serial No. 60/002,998, filed August 30, 1995.

FIELD OF THE INVENTION

This invention relates generally to transgenic animal models useful in testing agents for inhibition of CD8+ and CD4+ T-cell destruction of pancreatic beta cells. In particular, it relates to transgenic mice which have a T-cell receptor α and/or β chain (TCRβ) genes derived from beta cell cytotoxic CD8+ and/or CD4+ cells in their genome.

BACKGROUND OF THE INVENTION

The background of the present invention relates both to biological organisms which have been genetically transformed and to the mechanisms of diabetogenesis.

Genetic transformation of zygotes and the organisms resulting therefrom have been known for some time. Transgenic animals are those animals which carry a gene which has been introduced into the germline of the animal or its ancestor.

Insulin-dependent diabetes mellitus (IDDM) in humans and non-obese diabetic (NOD) mice results from a spontaneous, T-cell-dependent autoimmune process directed against pancreatic beta cells. At 4-5 weeks of age, NOD mice develop insulitis, followed by progressive destruction of the beta cells and, starting at 3 to 4 months of age, diabetes. About 60-90% of female and 10-30% of male NOD mice develop IDDM.

IDDM is a T-cell dependent disease.

In humans, T-cells are abundant among insulitis cells at IDDM onset, and IDDM recurs in diabetic patients treated with pancreas isografts or HLA-identical allografts. In NOD mice, most insulitis cells are also T cells and IDDM does not develop in athymic- or immunodeficient (*scid*)-NOD mice. Furthermore, IDDM can be prevented with anti-T cell reagents and can be transferred to immunodeficient or young NOD mice by T cells from diabetic mice. T cells are essential, but not sufficient; macrophages and/or dendritic cells also play an important role in IDDM.

CD4+ vs. CD8+ T cells as effectors of beta cell destruction in IDDM.

Most T cell transfer studies of NOD splenic T cells into NOD mice have concluded that IDDM transfer requires both CD4+ and CD8+ T-cells; however, the role of CD4+ vs. CD8+ T cells as effectors of beta cell destruction in spontaneous IDDM remains unclear. Adoptive T cell transfer experiments using beta cell-specific CD4+ T cell clones have shown that some, but not all clonotypes can destroy beta cells *in vivo* in the absence of CD8+ T cells. These adoptive T cell transfer studies have not determined, however, if, and to what extent, diabetogenic beta cell-specific CD4+ T cells also effect beta cell damage in the course of spontaneous IDDM. Indeed, expression of the T-cell receptor (TCR) rearrangements of a lymph node-derived diabetogenic CD4+ T cell clone in transgenic NOD mice increased the incidence of IDDM but did not accelerate its onset. These observations have suggested that beta cell specific CD4+ T cells may not function as major effectors of beta cell damage in spontaneous IDDM. Alternatively, activation of diabetogenic CD4+ T cells may require the shedding of beta cell autoantigens by a CD8+ T cell-mediated cytopathic effect on beta cells. This possibility is supported by two observations: splenic CD4+ T cells from pre-diabetic NOD mice can transfer

insulitis, but not IDDM, to *scid*-NOD mice, and MHC class-I/CD8+ T cell-deficient NOD mice do not develop IDDM.

The present inventor, and others, have provided evidence for the involvement of cytotoxic CD8+ T cells (CTL) as effectors of beta cell damage in IDDM. H-2K^d-restricted beta cell-specific CD8+ CTL are regularly present in islets of diabetic NOD mice, can transfer IDDM into irradiated NOD mice if co-injected with insulitogenic CD4+ T cells, and can kill beta cells of diabetes-resistant mice *in vivo*. However, as with similar studies with CD4+ T cell clones, these adoptive CTL transfer experiments have not determined if, and to what extent, beta cell-specific CD8+ T cells effect beta cell damage in *spontaneous* IDDM.

Previous investigations have generated strains of transgenic mice to investigate diabetogenesis or organ-specific autoimmunity in general. Expression of different transgenic antigens in islet beta cells of transgenic mice and/or TCR genes encoding receptors specific for these transgenic auto-antigens has resulted in several different outcomes (see references 31-43, below). Some of these transgenic models developed diabetes (see references 31 and 32, below) whereas others developed a non-immunologically-mediated form of hyperglycemia, possibly due to over-expression of the transgene (see references 33-34, below). In other models, the T cells bearing TCRs capable of recognizing the transgenic neo-antigen ("autoreactive") were tolerized (see references 35-37, below), or ignored the transgenic autoantigen (see references 38-43, below). There are several potential explanations as to why different antigens on the same tissue can induce autoimmunity or tolerance, or be ignored. The mechanisms that regulate the differentiation and function of thymocytes and peripheral T cells vary depending on the molecular nature of the antigen/TCR system studied (see references 44-46, below), as well as on the site of expression, amount, and timing of transgene expression during development (see references 44-50, below). In transgenic mice, the TCR

studied is also important, as TCR-transgenic mice generated with transgenes encoding TCR heterodimers with same specificity but different affinity show different susceptibility to autoimmunity and tolerance induction (see references 46. below).

Since neither the antigens nor the autoreactive T cells that were studied in these models are involved in spontaneous IDDM, and the genetic backgrounds in which these genes were expressed actually provide diabetes-resistance rather than susceptibility, it is difficult to determine which, if any, of the outcomes that were observed is IDDM-relevant. Thus, whether T cells recognizing disease-relevant non-transgenic beta cell autoantigens in NOD mice cause autoimmunity or undergo tolerance or ignorance in diabetes-resistant mice is unknown.

Recently, another group of investigators have generated transgenic NOD mice expressing the TCRα and TCRβ rearrangements of a diabetogenic beta-cell specific CD4+ T cell clone (see reference 51, below). However, expression of these TCR transgenes did not accelerate the onset of clinical diabetes.

References

The following references are representative of the state of the art with respect to transgenic animals and mechanisms of diabetogenesis. They describe in detail some currently known methods for producing transgenic organisms. They also disclose the current knowledge regarding mechanisms of diabetogenesis and, in particular, the role of CD4+ and CD8+ T-cells in diabetes.

1. U.S. Patent No. 4,736,866 for Transgenic non-human mammals, 1988.
2. U.S. Patent No. 4,873,191 for Genetic transformation of zygotes, 1989.

3. U.S. Patent No. 5,087,571 for Method for providing a cell culture from a transgenic non-human mammal, 1992.

4. U.S. Patent No. 5,174,986 for Method for determining oncogenic potential of a compound using transgenic mice predisposed to T-cell lymphomas, 1992.

5. U.S. Patent No. 5,175,384 for Transgenic mice depleted in mature T-cells and methods for making transgenic mice, 1992.

6. U.S. Patent No. 5,387,742 for Transgenic mice displaying the amyloid-forming pathology of Alzheimer's disease, 1995.

7. AU 9189231 for Transgenic rats and animal models of disease, 1992.

8. AU 9190761 for Mice having β2 microglobulin gene disruption, 1991.

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The disclosure of the above publications, patents and patent applications are herein incorporated by reference, as illustrative of the current knowledge of one skilled in the art, in their entirety to the same extent as if the language of each individual publication, patent and patent application were specifically and individually included herein.

In view of the above, it is evident that there is a need for an animal model to study the independent pathogenic effects of beta cell-specific CD4+ and CD8+ cells, and to develop/test therapies that interfere with the development and activation of these cells in vivo.

SUMMARY OF THE INVENTION

Previous investigators have generated strains of mice carrying TCR α and β genes from a diabetogenic CD4+ T cell clone isolated from an NOD mouse. The present inventor has unexpectedly found that the use of TCR α and/or β transgenes from CD4+ or CD8+ T cell clones isolated from the pancreatic islets of acutely diabetic NOD mice produces a useful transgenic animal model for diabetes, where the onset of the spontaneous diabetes is drastically accelerated.

The present invention provides transgenic animals which have cytotoxic CD4+ or CD8+ T-cell receptor alpha and/or beta chain genes in their genome (TCRα and/or TCRβ). The TCR-transgenic models of the present invention are unique in that: a) the target autoantigens are non-transgenic; b) the TCR specificities are disease-relevant as they were derived from cells involved in spontaneous diabetogenesis in non-transgenic NOD mice; and c) the T cells expressing the TCR transgenes cause a very early-onset destruction of beta cells that occurs in diabetes-prone, but not diabetes-resistant genetic backgrounds.

In one aspect, the invention provides a transgenic non-human mammal useful as a model for diabetes having incorporated into its genome a T-cell receptor beta chain gene derived from beta cell cytotoxic CD8+ and/or CD4+ cells.

In another aspect, the invention provides a transgenic non-human mammal useful as a model for diabetes having incorporated into its genome T-cell receptor alpha and beta chain genes from beta cell cytotoxic CD8+ or CD4+ T cells.

In another aspect, the invention provides a method for making an animal model useful for the study of diabetes by incorporating a T cell receptor α and/or β chain gene from a beta cell cytotoxic CD8+ or a beta cell cytotoxic CD4+ cell into the genome of a non-human mammal.

In yet another aspect, the invention provides a method for studying the effect of agents on diabetes comprising administering said agent to a transgenic non-human mammal having incorporated into its genome a T-cell receptor α and/or β chain gene from a beta cell cytotoxic CD8+ or a beta cell cytotoxic CD4+ cell, and monitoring the development of diabetes in said transgenic non-human mammal.

With the foregoing and other objects, advantages and features of the invention that will become hereinafter apparent, the nature of the invention may be more clearly understood by reference to the following detailed description of the preferred embodiments of the invention and to the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Generation of transgenic constructs used in the present invention.

Figure 2: CD4, CD8 and Vβ8.1/8.2 profiles of thymocytes (A) and lymph node cells (B) from transgenic mice generated with the TCRβ chain gene derived from a CD8+ T cell clone (NY8.3) and their non-transgenic littermates.

Upper panels show CD4 vs. CD8 dot plots of cell suspensions stained with anti-CD8-PE, anti-Vβ8.1/8.2-FITC, and anti-CD4-biotin plus Streptavidin-PerCP.

The lower panels show the Vβ8.1/8.2 fluorescence histograms of each T cell subset after electronic gating. Numbers indicate the average percentage of cells (upper panels) or the average number of Vβ8.1/8.2+ cells (lower panels) in each subset. DP, double positive cells; DN, double negative cells.

Figure 3: Proliferation of CD4+ T cell-depleted splenocytes from NY8.3 TCRβ-transgenic NOD mice in response to immobilized anti-CD3 and anti-Vβ8.1/8.2 mAbs.

Figure 4: Beta cell-specific T cells in TCRβ-transgenic NOD mice.

(A) Splenocytes from transgenic mice were either cultured with islets for 4 days, expanded with rIL-2 for 10 days, and restimulated with islets and rIL-2 (A, left), or depleted of CD4+ T cells, stimulated with plate-bound anti-CD3 mAb for 4 days, and expanded with rIL-2 for 10 days (A, right). Both T cell populations were analyzed by flow cytometry, and tested for cytotoxic activity against MIN6N8a and L929-Kd cells using ^{51}Cr-release assays.

(B) Anti-CD3-activated peripheral CD8+ T cells were tested for cytotoxic activity against MIN6N8a, L1210-Fas+ (L1210F+) and L1210-Fas- (L1210F-) cells before (B, left) and after (B, right) activation with PMA and ionomycin. This figure shows the average results of experiments with 2 different CD8+ T cell lines (± S.E.).

Figure 5: Limiting dilution analyses of beta cell specific precursor cytotoxic CD8+ T-cells (cytotoxic lymphocyte (CTL)) and islet-reactive CD4+ T-cells in NY8.3 TCRβ transgenic and non-transgenic mice.

Figure 6: NY8.3 TCRβ-transgene expression and diabetes. Cumulative incidence of diabetes in female (21 transgenic, 18 non-transgenic and 63 NOD/Lt) and male (30 transgenic, 32 non-transgenic and 39 NOD/Lt) mice. Diabetes was assessed by measuring urine glucose levels with Diastix strips twice a week . Animals were considered diabetic after two consecutive readings $\geq 3+$. *, p=0.0001-0.008 (females), or p=0.04-0.0004 (males) (Chi-square).

Figure 7: Insulitis, islet-infiltrating T cells and beta cell destruction in pre-diabetic NY8.3 TCRβ-transgenic and non-transgenic NOD mice.

A) Hematoxylin-eosin stained pancreatic sections of 3, 4, and 7 wk-old pre-diabetic, and diabetic transgenic and non-transgenic female mice (4-7 mice per age group; 20-30 islets/mouse) were scored for the degree of insulitis as described in Materials and Methods. *, $p<0.0001$ (Chi-Square).

B and C) Frozen sections from each pancreas were stained with anti-Mac-1+ (M1/70.15.11.5.HL), anti-CD8 (53.6-7) and anti-CD4 (GK1.5) mAbs, followed by FITC-labelled anti-rat IgG, and positive cells counted under a fluorescence microscope. Approximately 5 infiltrated islets (Mac-1+)/mouse were scored. *, $p<0.01$ (Mann-Whitney U).

D) Progression of beta cell loss, as determined by measuring the pancreatic insulin content of each of the pancreata studied above. *, $P<0.01$ (Mann-Whitney U).

E and F) Progression of activation of islet-associated CD4+ and CD8+ T cells in transgenic and non-transgenic female mice. Islets from individual transgenic and non-transgenic mice were purified, mechanically disrupted into single cell suspensions and analyzed by three-color flow cytometry using anti-CD4 FITC or anti-CD4-biotin, plus anti-CD8-PE and one of the following mAbs: anti-CD44-FITC; anti-CD25-FITC; anti-CD69-biotin; anti-CD62L-

biotin. Average results from 4-5 transgenic and non-transgenic NOD mice are shown.

Figure 8: Beta cell cytotoxic activity of islet-derived CD8+ T-cells from NY8.3 TCRβ-transgenic NOD mice. Purified islets from acutely diabetic TCRβ-transgenic NOD mice were cultured in the presence of rIL-2 for 5 d, depleted of CD4+ T cells by negative selection with GK1.5-coated immunobeads, stimulated for 7 d with irradiated NOD islets and rIL-2, analyzed by three-color flow cytometry using anti-CD8-PE, anti-CD4-biotin and anti-Vβ81./8.2-FITC (A), and tested for bet cell cytotoxic activity (B), serine esterase content (C) and target cell-induced cytotoxic granule exocytosis (D).

Figure 8A shows the FACS-transgenic profile of a representative islet-derived T cell line.

Figure 8B shows average results (x±S.E.) of experiments with 4 different islet-derived CD8+ T cell lines (P1, P3, P6 and 2910).

Figure 8C shows the total serine esterase content of islet-derived CD8+ T cells from a diabetic transgenic NOD mouse vs. anti-CD3-activated peripheral CD8+ T cells from the same mouse. The values represent the difference between the absorbance at 412 nm of non-lysed vs. lysed T cells.

Figure 8D shows the islet cell-induced % release of serine esterase activity from another islet-derived CD8+ T cell line from transgenic NOD mice.

Figure 9: Endogenous TCRα repertoire of islet-derived (A) and peripheral (B) CD8+ T cells from diabetic NY8.3 TCRβ-transgenic NOD mice. Total cellular RNA extracted from islet-derived T cells of 5 different diabetic transgenic mice, as well as from pooled CD4+ T cell-depleted splenic T cells from 2 diabetic transgenic mice was used to generate TCRα-specific cDNA libraries by anchor-PCR. Randomly-picked recombinants from each library

were then sequenced. Only the Vα-Jα junctional sequences of the different cDNAs found are shown. V-gene sequences were classified into families according to Wilson et al. (*Immunol. Rev.* 101:149 (1988)). Germline encoded Jα sequences are from Koop et al. (*Genomics* 13:1209 (1992)), or labelled as Jα...x. N is the ratio between the copy number of each cDNA and the number of cDNAs sequenced. N-terminal residues/nucleotides homologous to those of the TCRα chain of the TCRβ-transgene donor (CTL NY8.3) are underlined.

Va/Ja = TCRα-variable/joining genes; N = the number of copies/number of TCR cDNAs sequenced; Na = sequences encoded by N-terminal addition of nucleotides; CDR3 = complementary determining region 3. TCRα-CDR3 sequences that are homologous or identical to those of the CTL clone 8.3 are underlined in the figure.

Figure 10: CD4, CD8, and Vβ8.1/8.2 profiles of thymocytes (A) and splenocytes (B) from RAG-$2^{+/+}$ and RAG-$2^{-/-}$ NY8.3 TCRαβ-transgenic mice. See Fig 1 for details. Note that thymocyte development is skewed towards the CD8+ T cell subset in transgenic NOD mice expressing the TCR rearrangements of NY8.3.

Figure 11: The peripheral frequency of beta cell-specific CD8+ T cells in NY8.3 TCRαβ-transgenic vs. TCRβ-transgenic NOD mice. See Fig 5 for details.

Figure 12: The proliferation of bulk splenic CD8+ T cells from single- and double- transgenic NY8.3 mice in response to stimulation with NOD islets, in the presence (right) and absence (left) of exogenous rIL-2.

Figure 13: Cumulative incidence of diabetes in NY8.3 TCR$\alpha\beta$-transgenic (TG) female (n=14) and male (n=9) NOD mice and non-transgenic NOD mice (NON-TG). See Fig. 6 for details.

Figure 14: Phenotype of islet-derived T cells from acutely diabetic non-transgenic (A) and NY8.3 TCR$\alpha\beta$-transgenic (B) NOD mice. Pancreatic islets were cultured in rIL-2 and growing cells analyzed by FACS within 4 days. Note that most T cells from transgenic mice are CD8+ and Vβ8.1+.

Figure 15: CD4, CD8 and Vβ11 profiles of thymocytes (A) and splenocytes (B) from transgenic NOD mice generated with the TCRα and/or TCRβ chain genes derived from a CD4+ T cell clone (NY4.1). See Fig. 2 for details. NOD-NON-TG = non-transgenic NOD mice; NOD-TCRβ-TG= TCRβ transgenic NOD mice; NOD-TCR$\alpha\beta$-TG = TCR$\alpha\beta$ transgenic NOD mice.

Figure 16: Proliferation of bulk splenic CD4+ T cells from non-transgenic (NON-NOD-TG) and double transgenic NY4.1 mice (NOD-TG) in response to stimulation with NOD islets, in the absence of rIL-2.

Figure 17: Cumulative incidence of IDDM in NY4.1 TCR-transgenic (TG) and non-transgenic (NON-TG) NOD mice. Data correspond to 13 female transgenic mice and 13 male transgenic mice. See Fig. 6 for further details.

Figure 18: Phenotype of islet-derived T cells from acutely diabetic non-transgenic (NOD-NON-TG) (A) and NY4.1 TCR$\alpha\beta$ transgenic NOD mice (B). See Fig. 14 for further details. Note that most T cells derived from transgenic mice are CD4+ and Vβ11+.

Figure 19: Cytolytic activity and antigen-induced serine esterase release by islet-derived CD8+ T cells from diabetic NY8.3 TCR$\alpha\beta$-transgenic NOD mice. Figure 19A, without PMA and ionomycin; Figure 19B, following PMA and ionomycin activation (6h). Note that *in vivo*-activated CD8+ T cells from TCR$\alpha\beta$-transgenic NOD mice effect NOD islet-specific cytotoxic activity and secrete serine esterase in response to NOD islet cells (Fig. 19A). Upregulation of FasL on effector CD8+ T cells by stimulation with PMA and ionomycin results in CTL capable of killing irrelevant targets through Fas (Fig. 19B left), but not non-NOD beta cells (Fig. 19B right).

Figure 20: Cytolytic activity of islet-derived CD4+ T cells from NY4.1 TCR-transgenic NOD mice. Left-hand graph: cytolysis of islet cells from NOD mice in the presence (NOD/PMA/I) and absence (NOD i.c.) of PMA and ionomycin, and of islet cells from C57BL/6 mice in the presence (B6/PMA/I) and absence (B6 i.c.) of PMA and ionomycin. Right-hand graph: cytolysis of L1210-Fas$^+$ (L-Fas+/PMA/I) and L1210-Fas$^-$ (L-Fas-/PMA/I) in the presence of PMA and ionomycin.

DETAILED DESCRIPTION OF THE INVENTION

Beta cell-specific, H-2K^d-restricted TCR-transgenic NOD mice (NY8.3).

Islet-derived CD8+ CTL from diabetic NOD mice use highly homologous TCRα-antigen binding site (CDR3) sequences. Prior to the present invention, this suggested recognition of an immunodominant, autoantigen/H-2K^d complex on beta cells. The present inventor has shown that these beta cell-specific CD8+ T cells are major effectors of beta cell damage in spontaneous IDDM by generating transgenic NOD mice with the TCR rearrangements of a representative CTL clone. Expression of the TCRβ rearrangement of this clonotype in NOD mice causes a 10-fold increase in the precursor frequency of beta cell-specific CTL, and accelerates the onset of IDDM (by 5 wk in females

and by 7 wk in males). In these mice, IDDM onset is preceded by an accelerated recruitment of CD8+ CTL using endogenously-derived TCRα chains identical to that of the clonotype donating the TCRβ transgene to islets. Co-expression of the TCRα and TCRβ rearrangements of this CTL clone in NOD mice accelerates the onset of IDDM even further, with most mice becoming diabetic between 17 and 44 days of life. These results demonstrate that beta cell-specific CD8+ T cells accumulate in islets in response to beta cell antigens and that CD8+ CTL are major effectors of beta cell damage in spontaneous IDDM.

Studies of H-2I-A^{g7}-restricted beta cell-specific TCR$\alpha\beta$-transgenic NOD mice (NY4.1).

These observations in H-2K^{d}-restricted beta cell-specific TCR-transgenic NOD mice do not indicate that beta cell damage in IDDM is exclusively mediated by CD8+ T cells, or that beta cell-specific CD4+ T cells do not or cannot kill beta cells in IDDM. The observation that splenic CD4+ T cells from diabetic NOD mice can transfer IDDM to *scid*-NOD mice indicates that some splenic CD4+ T cell specificities are capable of destroying beta cells *in vivo* following adoptive T cell transfer; however, prior to the present invention is was not known whether these cells are also capable of destroying beta cells in spontaneous IDDM. The present inventor has generated transgenic NOD mice with the TCR rearrangements of an H-2I-A^{g7}-restricted beta cell-specific CD4+ T cell clone isolated from islets of a diabetic NOD mouse. IDDM onset in these mice is also accelerated, with most mice developing IDDM between 25 and 43 days of life. Comparison of these results with those obtained in the H-2I-A^{g7}-restricted TCR$\alpha\beta$-transgenic NOD mice of Katz et al., which did not develop early-onset IDDM, suggest that not all beta cell-specific CD4+ T cell clonotypes that are diabetogenic in adoptive T cell-transfer experiments are capable of effecting beta cell damage in *spontaneous* IDDM. The strikingly

different outcome of these two studies may be due to differences in the fine antigenic specificity of the transgenic TCRs. Note that while initiation of diabetogenesis in NOD mice coincides with an immunodominant CD4+ T cell response against glutamic acid decarboxylase, islet-associated CD4+ T cells of pre-diabetic NOD mice use heterogeneous TCRs and probably recognize multiple antigens; some of the CD4+ T cells recruited to islets during amplification of the immune response may not differentiate into effectors of beta cell damage *in vivo*.

The present invention provides transgenic animals which are uniquely suited for use as a mammalian model of autoimmune disorders, including autoimmune diabetes. In a preferred embodiment, the transgenic animals of the present invention are transgenic mice with beta-cell specific TCR that are naturally found in T cells, preferably CD4+ or CD8+ T cells, which may be isolated from the pancreatic islets of diabetic NOD mice. The transgenic animals of the present invention may have TCR which are transgenic in both the α and β chains of the receptor (TCR$\alpha\beta$), or in only the β chain (TCRβ).

The present invention thus provides TCRβ transgenic animals. In a preferred embodiment, the TCRβ transgenic animals are transgenic mice. Expression of a TCRβ transgene in the TCRβ transgenic animals of the present invention accelerates the development of IDDM, in a manner which is correlated with an increase in the peripheral frequency of precursor CTLs, and with the recruitment into islets of CTL using TCRα amino acid sequences which are at least homologous, and preferably identical to that of the CTL clone which donated the TCRβ transgene.

The present invention also provides TCR$\alpha\beta$ transgenic animals. In a preferred embodiment, the TCR$\alpha\beta$ transgenic animals are transgenic mice. Such TCR$\alpha\beta$ transgenic mice may be generated with beta cell-specific TCR of a CD4+ T cell clone, or with beta cell-specific TCR of a CD8+ T cell clone.

In a still more preferred embodiment, the TCR of a CD4+ T cell clone is a H-2I-A^{g7}-restricted beta cell specific TCR (Vβ11-Jβ2.4/Vαn.3-Jα33) of a CD4+ T cell clone isolated from the pancreatic islets of a diabetic NOD mouse. In another preferred embodiment, the TCR of a CD8+ T cell clone is a H-$2K^d$ restricted beta cell specific TCR (Vβ8.1-Jβ2.4/Vαn.1-Jα34). Expression of the TCR $\alpha\beta$ transgene in the transgenic animals of the present invention further accelerates the development of IDDM.

The present invention also provides a method for producing TCR transgenic mice which develop early-onset diabetes. In a preferred embodiment, this method comprises selecting genes of beta cell specific CD8+ or CD4+ T-cell clones derived from the pancreatic islets of diabetic NOD mice. In a preferred embodiment, the clones are NY8.3 (H-$2K^d$-restricted) and NY4.1 (H-2I-A^{g7}-restricted). The TCR genes are amplified, preferably by polymerase chain reaction (PCR). In a particularly preferred embodiment, the PCR will use primers carrying L-V or J-C intron sequences and convenient restriction sites at their 5' ends. The PCR products are then subcloned into a plasmid vector; in a preferred embodiment, the PCR products are pBluescript-SK+. The plasmid vector is then sequenced and inserted into TCRα and TCRβ shuttle vectors. These shuttle vectors preferably carry endogenous TCRβ or TCRα enhancers, respectively, and may further comprise 5' regulatory sequences. At this stage, prokaryotic sequences are preferably removed. Finally, the constructs are incorporated into the genome of an animal; in a preferred embodiment, the constructs are microinjected into fertilized eggs which are then implanted into the uteri of pseudopregnant females. In the case of mice, mouse (SJLxB6) F2 eggs are preferred. Founder animals are then crossbred for several generations, to generate TCR-transgenic animals in the desired genetic background.

The present invention also provides a method for evaluating agents which may be effective in arresting the development of IDDM. In the method

of the present invention, an agent to be evaluated is administered to the transgenic TCRβ or TCR$\alpha\beta$ transgenic mice of the present invention. The agent to be evaluated may be administered by any route known to the skilled artisan; preferably the agent to be evaluated is administered orally, parenterally, or intranasally. In one embodiment of the method present invention, administration of test agent and control compound begins perinatally; in this embodiment the percentage of mice which develop diabetes in the control versus the test group indicates the effectiveness of the test agent in preventing diabetogenesis. In an alternative embodiment of the method of the present invention, test and control compounds are administered to the transgenic mice of the present invention after development of diabetes. If the test agent reversed or alleviated symptoms of diabetes, that would indicate that the agent was useful for treating diabetes. The transgenic animals of the present invention provide a particularly useful test model due to the fact that they develop IDDM at an early age, which provides economic benefits for drug testing using this model. They are also a useful test model to evaluate the effectiveness of pharmacological or genetic agents in arresting the development of beta cell destruction that is effected by CD4+ or CD8+ T cells, respectively.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

Example 1: Preparation of Transgenic Mice

We generated transgenic NOD mice with the TCRα and/or TCRβ rearrangements of an H-2K^d-restricted beta cell-specific CTL clone (NY8.3) or of an H-2I-A^{g7}-restricted beta cell-specific CD4+ T cell clone (NY4.1), which were derived from islets of diabetic NOD mice.

A. Selection of transgenic specificities

We chose the T-cell clones NY8.3 (H-2K^d-restricted) and NY4.1 (H-2I-A^{g7}) for use as donors of TCR genes for the transgenic animals of the present invention. The NY8.3 clone was selected because: a) this clonotype was found to infiltrate pancreatic islets in 3 different NOD mice and uses a TCRα-CD3 sequence homologous to of most islet-derived CTL from non-transgenic NOD mice (see B, below), suggesting immunodominance; b) in addition to the functional TCRα (Vαn.1-Jα34) and TCRβ (Vβ8.1-Dβ2.1-Jβ2.4) cDNAs, out-of-frame TCRα and TCRβ transcripts from the second loci were also identified, thus ruling out expression of additional TCR chains; and c) this clonotype was diabetogenic *in vivo* (see C, below). The CD4+ T cell clone NY4.1, which recognizes a putative beta cell antigen in the context of the H-2I-A^{g7} molecule, expressed a Vβ11-Jβ2/Vαn.3-Jα33 TCR.

B. NY8.3 uses a TCRα-CDR3 sequence that is homologous to that of most islet-derived CTL.

To determine the repertoire of insulitis derived CD8+ CTL in NOD mice, we sequenced >200 TCRα and TCRβ cDNA sequences generated by anchor-PCR from CTL lines and clones derived from pancreatic islets of 10 different NOD mice. These CTL were oligoclonal but did not show skewed Vα, Vβ, Jα or Jβ gene usage when compared to CD8+ splenocytes.

However, unlike the TCRα rearrangements of splenic CD8+ T cells, most of the TCRα sequences cloned from islet-derived CTL (17/26) used highly homologous antigen-binding site (CDR3) sequences ($p<0.0001$). Since the TCRα locus is not allelically excluded, these data showed that these CTL, including NY8.3, recognize an immunodominant antigen/MHC complex on beta cells.

C. Independent pathogenic effects of beta cell-specific CTL in vivo.

Since splenic CD8+ T cells from diabetic mice and beta cell-specific CTL can only migrate into islets of NOD mice when co-injected with insulitogenic CD4+ T cells, the independent pathogenic effects of beta cell-specific CTL *in vivo* remained unclear. To determine this, we injected H-2K^d-restricted CTL clones NY8.3 or NY33.4 into STZ-induced diabetic (NODxBALB/c) or (NODxB6) F1 mice (diabetes-resistant backgrounds) that had received BALB/c (H-2^d) or B6 (H-2^b) islets, respectively, under the kidney capsule.

When injected 2-3 days (d) after transplantation, the CTL homed into the grafts within 2 d, recruited large numbers of host Mac-1+ and CD4+ and CD8+ T cells, and caused diabetes in 7/7 (NODxBALB/c) F1 mice. In contrast, none of the 4 (NODxB6) F1 mice which received H-2^b grafts and H-2K^d-restricted CTL, and none of the 8 (NODxBALB/c) F1 mice which received H-2^d grafts but not CTL, showed inflammation of the grafts or diabetes.

Depletion of CD4+ T cells in H-2^d islet-grafted (NODxBALB/c) F1 mice injected with CTL reduced the severity of, but did not preclude, CTL-induced graft damage in any of 5 mice. Therefore, we concluded that CTL have cytopathic effect on beta cells independent from CD4+ T-cells *in vivo*, that infiltration and destruction of grafted islets by CTL are MHC class I-restricted responses, and that CTL can recruit naive CD4+ T cells to the site leading to further beta cell damage.

D. Preparation of Transgenic Constructs

The rearranged TCR V(D)J genes of the beta cell specific CD8+ T-cell clone NY8.3 (H-2K^d-restricted) and the beta cell-specific CD4+ T cell clone NY4.1 (H-2I-A^{g7}-restricted) were amplified by polymerase chain reaction (PCR) using primers carrying L-V or J-C intron sequences and convenient restriction sites at their 5' ends. The PCR products were subcloned into Bluescript-SK+,

sequenced and inserted into TCRα and TCRβ shuttle vectors carrying endogenous TCRβ or TCRα enhancers, respectively, and 5' regulatory sequences (3A9β and PRE53α/AN6α, provided by Dr. M Davis (Stanford Univ.) and S. Hedrick (UCSD)) (Figure 1). After removing prokaryotic sequences, the constructs (TCRβ and/or TCRα) were microinjected together (TCRα+β) or alone (TCRβ) into fertilized mouse (SJLxB6) F2 eggs, which were implanted into the uteri of pseudopregnant females.

We chose (SJLxB6) F2 mouse eggs because expression of this specificity in a NOD background might have caused early onset IDDM and breeding problems. Founder mice were crossed with NOD mice for several generations, to generate TCR-transgenic NOD mice. All mice were kept in specific pathogen-free conditions.

Example 2: CD4, CD8 and Vβ8.1/8.2 Profiles of Thymocytes and Lymph Node Cells from NY8.3 TCRβ Transgenic Mice

Thymocytes (Figure 2A) and lymph node cells (Figure 2B) from NY8.3 TCRβ transgenic mice and non-transgenic littermates (n=3-4) were stained with anti-CD4-biotin, anti-CD8-phycoerythrin (PE) and anti-Vβ8.1/8.2-fluorescein isothiocyanate (FITC) monoclonal antibodies followed by Streptavidin-PerCP. Samples were analyzed using fluorescence activated cell sorting (FACS) with a FACScan.

The upper panels of the Figures show dot plots of CD4 versus CD8. The lower panels show the fluorescence histograms for Vβ8.1/8.2 of each thymocyte (Figure 2A) and lymph node T-cell (Figure 2B) subset. Numbers correspond to the average percentage of cells expressing the corresponding markers, and demonstrate that >85% of single positive thymocytes and peripheral T-cells from TCRβ transgenic mice expressed the Vβ8.1 specificity, compared to <18% of non-transgenic mice, even though the absolute and relative numbers of the different thymocyte and peripheral T-cell subsets and

the cell surface densities of the TCR$\alpha\beta$ molecules and associated co-receptors on each type of cell were similar in transgenics and non-transgenics. (Figures 2A and 2B).

Example 3: Proliferation of CD4+ T-cell Depleted Splenocytes from NY8.3 TCRβ-Transgenic NOD Mice in Response to Immobilized Anti-CD3 and Anti-Vβ8.1/8.2 Monoclonal Antibodies

To determine whether the TCRβ transgene was appropriately expressed on CD8+ T cells of transgenic animals, we assessed the ability of CD4+ T cell-depleted splenocytes from transgenic and non-transgenic mice to proliferate in response to plate-bound anti-Vβ8.1/8.2 or anti-CD3 monoclonal antibodies (mAbs) and interleukin-2 (IL-2).

CD4-depleted splenocytes (7×10^4) from transgenic mice or non-transgenic littermates were incubated for 72 hours (h) in ELISA-grade 96 well plates which were precoated with serial dilutions of the anti-Vβ8.1/8.2 monoclonal antibody (mAb) KJ16, which had been ammonium sulfate cut from ascites fluid, and affinity purified anti-CD3 mAb 2C11. The cultures were pulsed with 1 μCi of ^{3}H-thymidine for 18 h before harvesting. ^{3}H-thymidine incorporation was assessed by scintillation counting.

As shown in Figure 3, T cells from transgenic mice proliferated much more efficiently than those from non-transgenic mice in response to anti-Vβ8.1/8.2, whereas proliferation in response to anti-CD3 was comparable in transgenic and non-transgenic animals. Accordingly, these results demonstrate that the TCRβ transgene was appropriately expressed on CD8+ T-cells of transgenic animals.

Example 4: Beta Cell Specificity/Cytotoxicity of Peripheral T-Cells from NY8.3 TCRβ Transgenic Mice

To confirm that the transgenic TCRβ chain could confer beta cell-

specificity and that such specificity was predominantly expressed on CD8+ T cells, we stimulated splenic T cells from transgenic mice with irradiated islets followed by IL-2.

Splenocytes from transgenic mice were either:

(a) depleted of CD4+ T-cells by two rounds of negative selection with anti-CD4 mAb GK1.5 and goat anti-rat IgG-coated magnetic beads and grown in wells coated with anti-CD3 mAb for 4 days (d), followed by treatment with recombinant IL-2 (rIL-2) for 10 d; or

(b) cultured (2×10^5 cells) with 50 irradiated (2,000 rad) male NOD mouse islets for 4 d, expanded with rIL-2 for 10 d and restimulated with islets and IL-2.

Proliferating cells were analyzed by FACS, using anti-CD8-PE and anti-Vβ8.1/8.2-FITC, and tested for islet cytotoxicity in ^{51}Cr-release assays using NOD mouse-derived MIN6N8a insulinoma cells or H-2K^d-transfected L929 fibroblasts (L929-Kd) as target cells.

As shown in Figure 4, this protocol resulted in the preferential expansion of CD8+ T cells, which killed MIN6N8a insulinoma cells, but not H-2K^d-transfected L929 fibroblasts (L929-Kd). We showed that this expansion was induced by islets, because stimulation of CD4+ T cell-depleted splenocytes with plate-bound anti-CD3 mAb and IL-2 resulted in cells able to kill L929-Kd cells, but not MIN6N8a cells (Figure 4). The killing of L-929-Kd cells by polyclonally activated CD8+ T cells from TCRβ-transgenic mice was mediated through a non-antigen-specific and non-H-2K^d-restricted mechanism, because they were also able to kill P815 (H-2^d) and EL-4 (H-2^b) lymphoma cells (data not shown). Since these anti-CD3-activated CD8+ T cells were also cytopathic for Fas cDNA-transfected L1210 cells, but not for Fas-negative L1210 cells, it is likely that their non-antigen/MHC-specific cytotoxic activity was mediated through Fas (Fig 4B, left). The inability of these cytotoxic CD8+ T cells to kill MIN6N8a cells, even after upregulation of FasL expression by stimulation

with PMA and ionomycin, indicates that MIN6N8a cells are relatively resistant to Fas-mediated cytotoxicity (Fig 4B, right).

Thus, when expressed on CD8+ T cells, the transgenic TCRβ chain can combine with endogenous TCRα chains to form beta cell-specific TCRs on CD8+ T cells. The data further show that these cells can be induced to proliferate and differentiate into beta cell-cytotoxic effectors *in vitro* by stimulation with NOD islets.

Example 5: Limiting Dilution Analyses of Beta Cell-Specific Precursor CTL and Islet-Reactive CD4+ T-Cells in NY8.3 TCRβ Transgenic and Non-transgenic Mice

Twelve replicate cultures of serial dilutions of spleen cells from transgenic mice and non-transgenic littermates (groups of 2 mice each; same % of CD8+ and CD4+ T-cells) were stimulated with or without irradiated NOD islets as in Example 4. Cultures were tested for their ability to kill MIN6N8a or L929-Kd cells in ^{51}Cr-release assays conducted 7-10 d after the second stimulation with islets. Cultures were considered positive if they were able to kill MIN6N8a cells but not L929-Kd cells. The CTL frequencies were calculated by Poisson statistics.

The results, shown in Figure 5, show that NY8.3 TCRβ transgenic mice had approximately 10 times the number of beta cell-specific pre-CTL as non-transgenic mice.

To determine the peripheral frequency of islet cell-reactive CD4+ T-cells in these mice, we prepared CD8+ T-cell depleted splenocytes from transgenic and non-transgenic mice using anti-CD8 mAb 53-6.7 and goat anti-rat IgG-coated magnetic beads. We then cultured 12 replicate cultures of serial dilutions of CD8+ T-cell depleted splenocytes with 2,500 islet cells and 2 x 10^5 irradiated NOD mouse splenocytes per well or with irradiated NOD mouse splenocytes alone. None of the cultures that did not receive islet cells

proliferated, and the percentage of growing wells in islet stimulated cultures was proportional to the responder cell input. Results of this experiment revealed that the precursor frequency of islet-reactive CD4+ T cells in transgenic and non-transgenic mice were similar, as expected. Therefore, when compared with non-transgenic NOD mice, TCRβ-transgenic NOD mice have a selective increase in the peripheral frequency of beta cell-specific precursor CTL, but not an increase in the absolute or relative number of peripheral CD8+ T cells at the expense of CD4+ T cells, or in the frequency of islet-reactive CD4+ T cells.

Example 6: Cumulative Incidence of Diabetes in TCRβ Transgenic and Non-transgenic NOD/Lt Mice

To determine whether the observed increase in the frequency of pre-CTL had pathogenic significance, we screened transgenic and non-transgenic mice for development of IDDM. IDDM was monitored by measuring urine glucose twice per week. Mice were considered diabetic after two consecutive 3+ glucose readings.

The results in Figure 6 demonstrate the onset of diabetes was accelerated in transgenic mice as compared to non-transgenic littermates or NOD/Lt mice. The average age for onset of diabetes in transgenic mice was significantly lower than in non-transgenic mice (12.6 $\pm$ 1.7 weeks of age vs. 17.1 $\pm$ 3.9 weeks of age for females ($p<0.0001$) and 15.6 $\pm$ 4.7 weeks of age vs. 23.1 $\pm$ 4.0 weeks of age for males ($p<0.0002$)). However, the cumulative incidence of IDDM by 32 wk was similar in transgenic and non-transgenic NOD mice (85.7% vs. 87.3% for females and 50% vs. 51.3% for males).

All transgenic female NOD mice which became diabetic (85.7%) did so between 9-15 weeks of age. By contrast, IDDM in non-transgenic female littermates and NOD/Lt females only affected 27.8-31.7% of mice by 15 weeks of age ($p<0.0002$).

Among males, IDDM also started earlier in transgenics than in non-transgenics (11 weeks of age vs. 15 weeks of age); by 17 weeks of age, it already affected 33.3% of transgenic, but only 3-5% of non-transgenic and NOD/Lt mice, respectively ($p<0.003$).

Example 7: Insulitis, Islet-infiltration by CD4+ and CD8+ T cells and Beta Cell Destruction in Pre-Diabetic NY8.3 TCRβ-Transgenic NOD Mice

To investigate the mechanisms underlying disease acceleration in TCRβ-transgenic NOD mice, we followed the progression of insulitis and beta cell destruction in pre-diabetic animals. The degree of insulitis in 3 to 5 wk-old transgenic and non-transgenic mice was similar (Fig. 7A). At these ages, most of the infiltrated islets (>55%) displayed only peri- or mild insulitis and contained Mac-1+ cells, but very few or no CD8+ or CD4+ T cells (Fig. 7B, C). In contrast, the degree of insulitis in 7 wk-old transgenic mice was more severe than in their non-transgenic littermates (insulitis score: 2.7 ± 0.3 vs. 1.9 ± 0.2), owing not to a greater number of inflamed islets, but to an increased percentage of islets with severe inflammation (24.2% vs. 6.5%, $p<0.000\ 1$) (Fig. 7A). Faster progression of insulitis in transgenic mice was attributable to faster recruitment of CD8+ T cells (95.8 ± 42.3 vs. 28.7 ± 8.2 cells/islet, $p<0.01$), but not of CD4+ T cells (244 ± 64.7 vs. 197 ± 137 cells/islet) to inflamed islets (Fig. 7B, C), and resulted in an earlier onset and faster progression of beta cell depletion, as determined by comparing the pancreatic insulin content of transgenic vs. non-transgenic mice ($p<0.01$, Fig. 7D). Faster progression of beta cell depletion in transgenic mice was not a result of accelerated activation of islet-infiltration CD4+ T cells by local CD8+ T cells, or vice versa, since no differences were noted between the percentage of islet-associated CD4+ or CD8+ T cells expressing CD25, CD44, CD49d, CD62L and CD69 in transgenic vs. non-transgenic mice (Fig. 7E, F).

Example 8: Cytotoxicity of Islet-Derived CD8+ T-Cells from NY8.3 TCRβ-Transgenic NOD Mice

To determine whether the islet-associated CD8+ T cells of TCRβ-transgenic NOD mice had differentiated into β-CTL, we determined the cytotoxic activity of CD8+ T cell lines isolated from the pancreatic islets of 5 different acutely diabetic TCRβ-transgenic NOD mice. As shown in Fig. 8, islet-derived CD8+ T cells from diabetic mice were >98% Vβ8+ and displayed specific cytotoxic activity against beta cells, but not against irrelevant targets (L929-Kd in Fig. 8). Experiments with one of these islet-derived β-CTL lines (P1) confirmed that these cells were also cytotoxic to beta cells *in vivo*; like the parental cloning NY8.3, they could destroy BALB/c islet grafts (H-2K^{d+}) in streptozotocin- induced diabetic (NOD x BALB/c) F1 mice (data not shown).

Example 9: Endogenous T-Cell Receptor Alpha Chain Rearrangements of Islet-Derived CTL Lines from NY8.3 TCRβ-Transgenic NOD Mice

To determine whether the faster accumulation of beta cell-specific CD8+ CTL in islets of TCRβ-transgenic NOD mice was driven by stimulation with immunodominant beta cell autoantigens, as opposed to multiple autoantigens, non-antigen specific stimuli or superantigens, we determined the endogenous TCRα repertoire of each of these 5 islet-derived CD8+ T cell lines by sequencing randomly-picked recombinants from TCRα-specific cDNA libraries generated by anchored-PCR. Unlike islet-derived beta cell-cytotoxic CD8+ T cells from non-transgenic NOD mice, which use homologous TCRα-CDR3 sequences, but heterogeneous Vα, Vβ, Jα and Jβ elements [23], the predominant TCRα cDNAs of 4 of these 5 different CD8+ T cell lines from TCRβ-transgenic NOD mice encoded an amino acid sequence identical to that of the TCRα rearrangement used by the β-CTL clone donating the TCRβ transgene (Fig. 9). These predominant TCRα sequences were likely derived

from more than one clonotype, since their N-terminal residues were encoded by codons carrying silent nucleotide substitutions. The degree of clonality within each of these four individual lines cannot be precisely defined, however, because most silent substitutions between TCRα sequences of different CD8+ T cell lines were restricted to the third base of the codon encoding the Asp residue of the TCRα-CDR3 region, and there was a preference for GAT over GAC. The TCRα cDNAs of 1 of the 5 T cell lines that were studied encoded a CDR3 sequence homologous to that used by most islet-derived β-CTLs from non-transgenic NOD mice, including NY8.3, containing a core motif composed of a hydrophobic residue followed by arginine. These data therefore indicate that islet-associated CD8+ T cells from transgenic mice are beta cell-cytotoxic and that their accumulation in islets of NOD mice is driven by stimulation with a putative immunodominant beta cell autoantigen/H-2K^d complex.

Using the transgenic mice of the present invention, we have found that there is a selective increase in the precursor frequency of beta cell-specific CTL in transgenic NOD mice. This led to accelerated onset of IDDM, without increasing its incidence or accelerating the onset or progression of insulitis, owing to accumulation of CTL within islets using TCRα chains identical to that of the CTL clone which donated the TCRβ transgene.

Example 10: Double TCR$\alpha\beta$-Transgenic NOD Mice (NY8.3)

In transgenic NOD mice expressing the TCRα and TCRβ rearrangements of NY8.3, thymocyte development is skewed towards the CD8+ T cell subset (Fig. 10, left). Both TCR transgenes are expressed appropriately because most single-positive thymocytes and peripheral T cells are Vβ8+, and RAG-2$^{-/-}$ TCR$\alpha\beta$-transgenic NOD mice (unable to rearrange endogenous TCR genes) contain abundant CD8+, but no CD4+ T cells (Fig. 10, right). In RAG$^+$ TCR$\alpha\beta$-transgenic NOD mice, most splenic CD8+ T cells are beta cell-specific; they secrete as much as 1,000 pg/ml of TNF-α in

response to beta cells, but <1 pg/ml in response to control targets (Table I), and the frequency of splenic CD8+ T cells that proliferate *in vitro* in response to NOD islets is $\approx$400 times greater than in non-transgenic mice and $\approx$40 times greater than in TCRβ-transgenic mice ($\approx$1/17) (Fig. 11). Furthermore, bulk splenic CD8+ T cells from double-, but not single-, transgenic mice proliferate efficiently in response to stimulation with NOD islets both in the absence and presence of exogenous rIL-2, suggesting T-helper cell independence for proliferation (Fig. 12).

TABLE I. Secretion of TNFα by splenic and islet-derived CD8+ T cells from TCR-transgenic NOD mice (pg/ml)

Effector	Target	
	MIN6N8a	L929-Kd
Naive		
TCR$\alpha\beta$-tg	<1	<1
TCRβ-tg	<1	<1
α-CD3-activated*		
TCR$\alpha\beta$-tg	300	<10
TCRβ-tg	<1	<10
Islet-derived**		
TCR$\alpha\beta$-tg	1,000	<1
TCRβ-tg	250	<1

CD8+ T cells ($1X10^5$) were cultured with NOD-derived insulinoma cells (MIN6N8a) or H-$2k^d$-transfected L929 cells (L929-Kd) (1×10^4) for 24h. The concentration of TNFα in the supernatants was determined with a bioassay using WEHI clone 14 cells as indicators.
*activated with plate-bound anti-CD3 mAb (10 μg/ml) for 3 d and expanded with rIL2 for 7 d.
**from diabetic mice within 3 d of diabetes onset.

TCR$\alpha\beta$-transgenic NOD mice develop IDDM much earlier than TCRβ-transgenic and non-transgenic NOD mice; the incidence of diabetes in N3-N4 mice is 79% for females (11/14) and 33% for males (3/9), with an average age

at onset of IDDM of 39 ± 4.2 days (Fig. 13). The peripheral beta cell-specific CD8+ T cells of diabetic transgenic mice do not show signs of activation, as determined by FACS analysis with mAbs specific for activation and memory markers (CD25, CD44, CD62L and CD69), suggesting that they do not undergo activation in the periphery (data not shown).

Immunopathological studies of acutely diabetic transgenic NOD mice revealed severe islet infiltration by both CD4+ and CD8+ T cells. However, expansion of islet-associated T cells from acutely diabetic transgenic animals yields >90% CD8+ T cells, as compared to ≈50% in non-transgenic NOD mice. These cells are beta cell-specific because they secrete TNF-α in response to beta cells, but not control targets (Table I), and kill NOD (H-2K^{d+}), but not C57BL/6 (H-2K^{d-}), beta cells *in vitro* (see Example 12, below).

Example 11: H-2I-A^{g7}-Restricted TCR-Transgenic NOD Mice (NY4.1)

In transgenic NOD mice expressing the TCR rearrangements of the beta cell-specific CD4+ T cell clone NY4.1, most single-positive thymocytes and peripheral T cells, which proliferate in response to NOD islet cell antigen *in vitro* in the absence of exogenously added rIL-2 (Fig. 16), express the transgene-encoded Vβ11 element (Fig. 15A and B). Limiting dilution analyses using CD8+ T cell-depleted splenocytes suggest that in these mice the frequency of peripheral CD4+ T cells able to proliferate in response to stimulation with NOD islets and irradiated splenocytes is >1/250, ≈160 times greater than in non-transgenic NOD mice (data not shown).

Expression of this H-2I-A^{g7}-restricted beta cell-specific TCR in transgenic NOD mice also accelerates the onset of IDDM without increasing the overall incidence of the disease. The incidence of diabetes in N3-N5 transgenic mice is 77% in females (10/13) and 54% in males (7/13). The age at onset of diabetes is 38 ± 9 days in females and 47 ± 20 days in males (Fig 17). The CD4+ T cells of diabetic transgenic mice do not appear to undergo activation

in the periphery (data not shown). While the islet mononuclear infiltrates of diabetic transgenic mice contain abundant CD4+ and CD8+ T cells, most T cells recovered from islets cultured in rIL-2 are CD4+ (Vβ11+) (Fig. 18), as compared to $\approx$50% in non-transgenic NOD mice or <4% in H-2K^d-restricted TCR$\alpha\beta$-transgenic NOD mice (Fig. 14 and 18). The islet-derived CD4+ T cells of these mice are diabetogenic because they can transfer diabetes into *scid*-NOD mice (1/1 T cell-transferred mouse has become diabetic with all islet-infiltrating T cells being CD4+; data not shown).

Example 12: Cytolytic Activity of Transgenic CD8+ and CD4+ T Cells from NY8.3 and NY4.1 TCR$\alpha\beta$-Transgenic NOD Mice, Respectively

Indirect evidence suggests that the cytolytic activity of islet-derived CD8+ T cells from H-2K^d-restricted transgenic NOD mice (NY8.3) is mediated through perforin; these cells kill NOD, but not C57BL/6 islet cells (Fig 19A, left), even after upregulation of cell-surface FasL by stimulation with PMA and ionomycin (Fig. 19B, right). Furthermore, these cells specifically secrete serine esterase activity into the supernatant in response to stimulation with NOD, but C57B46 islet cells (Fig. 19A, right). In contrast, the islet-associated CD4+ T cells of H-2I-A^{g7}-restricted TCR-transgenic NOD (NY4.1) mice kill neither NOD nor C57BL/6 islet cells *in vitro* (Fig. 20, left). This is not surprising, since beta cells do not normally express the restricting H-2I-A^{g7} molecule. However, stimulation of these CD4+ T cells with PMA and ionomycin endows them with the ability to kill both NOD and C57BL/6 islet cells (Fig. 20, right). The cytolytic activity of these CD4+ CTL appears to be mediated through Fas, because they can also kill L1210-Fas$^+$, but not L1210-Fas$^-$ cells (Fig. 20, right). Thus, these beta cell-specific CD4+ T cells can kill islet cells through a non-MHC-restricted and Fas-dependent mechanism.

Example 13: Diabetes in CD4+ and CD8+ T Cell Monoclonal NOD Mice

To determine whether NOD mice carrying a monoclonal immune system also develop diabetes, we generated H-2K^d-restricted and H-2I-A^{g7}-restricted TCR$\alpha\beta$-transgenic NOD mice deficient in RAG-2; these mice are unable to rearrange endogenous TCR and Ig genes and as a result have no mature B or T lymphocytes other than those expressing the transgenic TCRs. Surprisingly, RAG-2-deficient H-2I-A^{g7}-restricted TCR-transgenic NOD mice which only have CD4+ Vβ11+ T cells in the periphery develop diabetes with an incidence and an age at onset of diabetes similar to those observed in RAG-2-positive mice (incidence of 60% and age at onset of 40 $\pm$ 18 days for females and incidence of 40% and age at onset of 39 $\pm$ 15 days for males). As expected, virtually all islet-infiltrating T cells of these mice are CD4+. These results indicate that these CD4+ T cells can spontaneously differentiate into effectors of beta cell damage *in vivo* in the absence of other T or B lymphocytes.

RAG-2-deficient NOD mice expressing the H-2K^d-restricted TCR have also been generated. All peripheral T cells of these mice are CD8+Vβ8.1+, as expected (Fig. 10, right). One female studied has developed diabetes, albeit significantly later than in RAG-2-positive mice (15 weeks), with virtually all islet-infiltrating T cells being CD8+. Another cohort of mice are now being followed for diabetes development. It appears that these mice do not develop diabetes at an early age. Altogether, the data suggests that differentiation can occur in the absence of endogenous T or B lymphocytes but progresses much faster in their presence.

The early onset of spontaneous diabetes in mice bearing monoclonal T cell immune repertoires makes them perfect models to test as well as to understand the mechanisms of action of known and novel immunotherapeutic and/or immunoprophylactic protocols aimed at curing and/or preventing organ-specific autoimmune diseases in general, and diabetes in particular.

Modification of the above-described modes of carrying out various embodiments of this invention will be apparent to those skilled in the art following the teachings of this invention as set forth herein. The examples described above are not limiting, but are merely exemplary of this invention, the scope of which is defined by the following claims.

WHAT IS CLAIMED IS:

1. A transgenic non-human mammal useful as a model for early-onset diabetes having incorporated into its genome T-cell receptor genes from beta cell cytotoxic T cells.

2. The transgenic mammal of claim 1, wherein the genes comprise TCR beta chain genes derived from CD8+ T cells.

3. The transgenic mammal of Claim 2 wherein said beta chain gene are the TCRβ genes of CTL clone NY8.3 (H-2K^d-restricted).

4. The transgenic mammal of Claim 2, wherein the genes further comprise TCR alpha chain genes derived from CD8+ T cells.

5. The transgenic mammal of Claim 4 wherein said alpha chain genes are the TCRα genes of CTL clone NY8.3 (H-2K^d-restricted).

6. The transgenic mammal of claim 1, wherein the genes comprise TCR alpha and beta chain genes derived from CD4+ T cells.

7. The transgenic mammal of claim 6, wherein said alpha and beta chain genes are TCR genes of CD4+ clone NY4.1 (H-2I-A^{g7}-restricted).

8. The transgenic mammal of Claim 1 wherein said mammal is a mouse.

9. The transgenic mammal of Claim 8 wherein said mouse is an NOD mouse.

10. A method for producing TCR transgenic mammals, comprising:
selecting genes of pancreatic islet-derived beta cell specific T cell clones;
amplifying the selected genes;
subcloning the amplification products into a vector; and
incorporating the vector into the genome of a mammal.

11. The method of claim 10, wherein the beta cell specific T cell clones are derived from pancreatic islets of diabetic NOD mice.

12. The method of claim 10, wherein the beta cell specific T cell clones are NY8.3 (H-2K^{d}-restricted) and NY4.1 (H-2I-A^{g7}).

13. The method of claim 10, wherein the selected genes are amplified by polymerase chain reaction.

14. The method of claim 13, wherein the PCR uses primers carrying L-V or J-C intron sequences and convenient restriction sites at their 5' ends.

15. The method of claim 10, wherein the vector is a plasmid vector.

16. The method of claim 15, wherein the plasmid vector is pBluescript-SK+.

17. The method of claim 15, wherein after the amplification products are subcloned into the plasmid vector, the plasmid vector is sequenced and inserted into TCRα and TCRβ shuttle vectors.

18. The method of claim 17, wherein the shuttle vectors further comprise endogenous TCRβ or TCRα enhancers and 5' regulatory sequences.

19. The method of claim 17, wherein prokaryotic sequences are removed from the shuttle vector prior to incorporation into the genome of the mammal.

20. The method of claim 10, wherein the vector is microinjected into fertilized eggs, which are then implanted into the uteri of pseudopregnant females.

21. The method of claim 20, wherein the mammals are mice, and the eggs are mouse (SJLxB6) F2 eggs.

22. A method for evaluating agents which may be effective in arresting the development of IDDM, comprising administering an agent to be evaluated to the transgenic TCRβ or TCR$\alpha\beta$ transgenic mammals of the present invention, and evaluating the effectiveness of the agent in preventing diabetogenesis.

23. The method of claim 22, wherein the agent to be evaluated is administered orally, parenterally, or intranasally.

24. The method of claim 22, wherein administration of test agent and control compound begins perinatally.

25. A method for evaluating agents which may be effective in the treatment of IDDM, comprising administering an agent to be evaluated to the transgenic TCRβ or TCR$\alpha\beta$ transgenic mammals of the present invention after the development of diabetes in those mammals, and evaluating the effectiveness of the agent in reversing or alleviating the symptoms of diabetes.

26. A method for evaluating gene therapy which may be effective in arresting the development of IDDM, comprising administering the gene therapy to be evaluated to the transgenic TCRβ or TCR$\alpha\beta$ transgenic mammals of the present invention, and evaluating the effectiveness of the gene therapy in preventing diabetogenesis.

27. The method of claim 26, wherein administration of gene therapy begins perinatally.

28. A method for evaluating gene therapy which may be effective in the treatment of IDDM, comprising administering gene therapy to be evaluated to the transgenic TCRβ or TCR$\alpha\beta$ transgenic mammals of the present invention after the development of diabetes in those mammals, and evaluating the effectiveness of the gene therapy in reversing or alleviating the symptoms of diabetes.

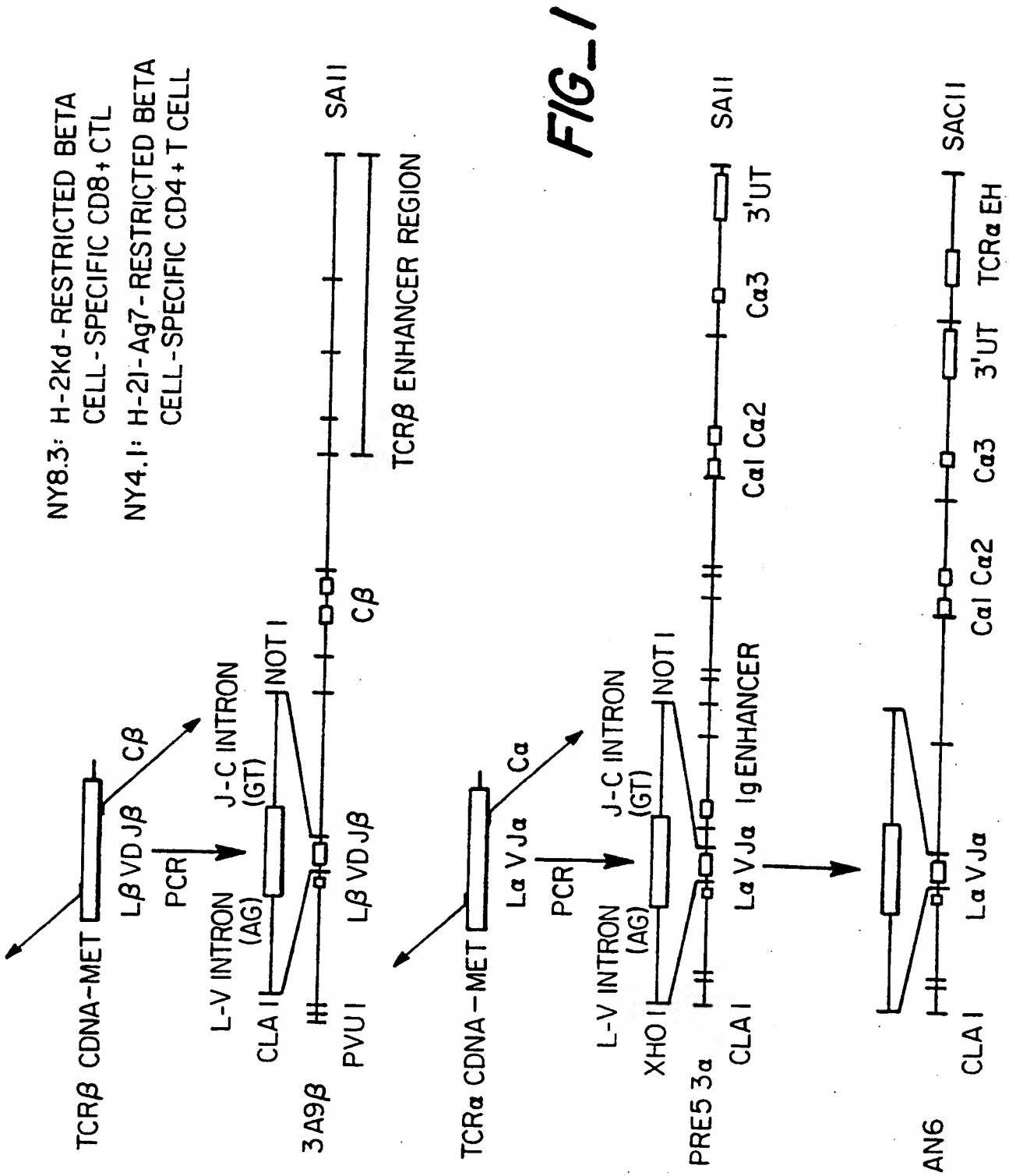
NY8.3: H-2Kd-RESTRICTED BETA CELL-SPECIFIC CD8+ CTL
NY4.1: H-2I-Ag7-RESTRICTED BETA CELL-SPECIFIC CD4+ T CELL
TCRβ CDNA-MET
Lβ VD Jβ
Cβ
PCR
L-V INTRON (AG)
J-C INTRON (GT)
NOT I
CLA I
PVU I
3A9β
Lβ VD Jβ
Cβ
SAI I
TCRβ ENHANCER REGION
TCRα CDNA-MET
Lα VJα
Cα
PCR
L-V INTRON (AG)
J-C INTRON (GT)
NOT I
XHO I
PRE5 3α
CLA I
Lα VJα Ig ENHANCER
Cα1 Cα2
Cα3
3'UT
SAI I
AN6
CLA I
Lα VJα
Cα1 Cα2
Cα3
3'UT
TCRα EH
SAC I

FIG_1

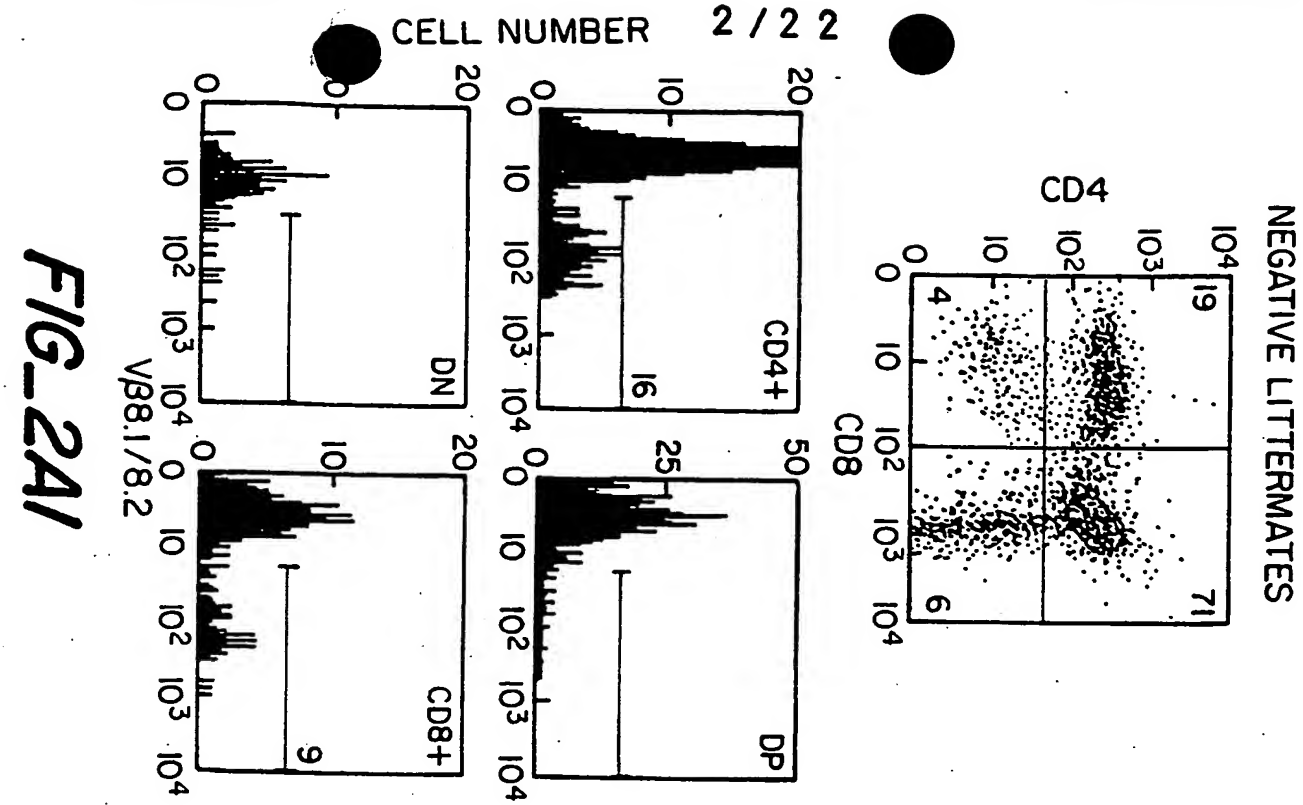

FIG_241

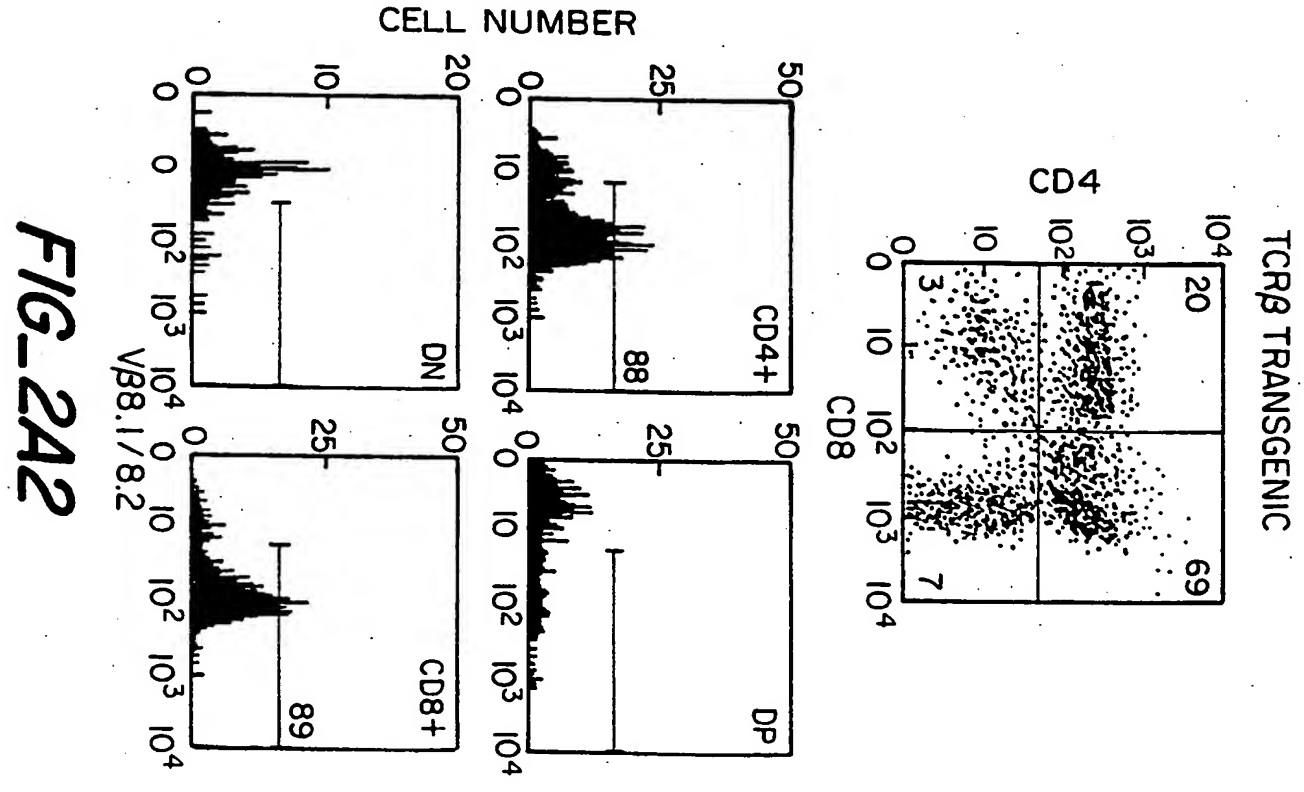

FIG_242

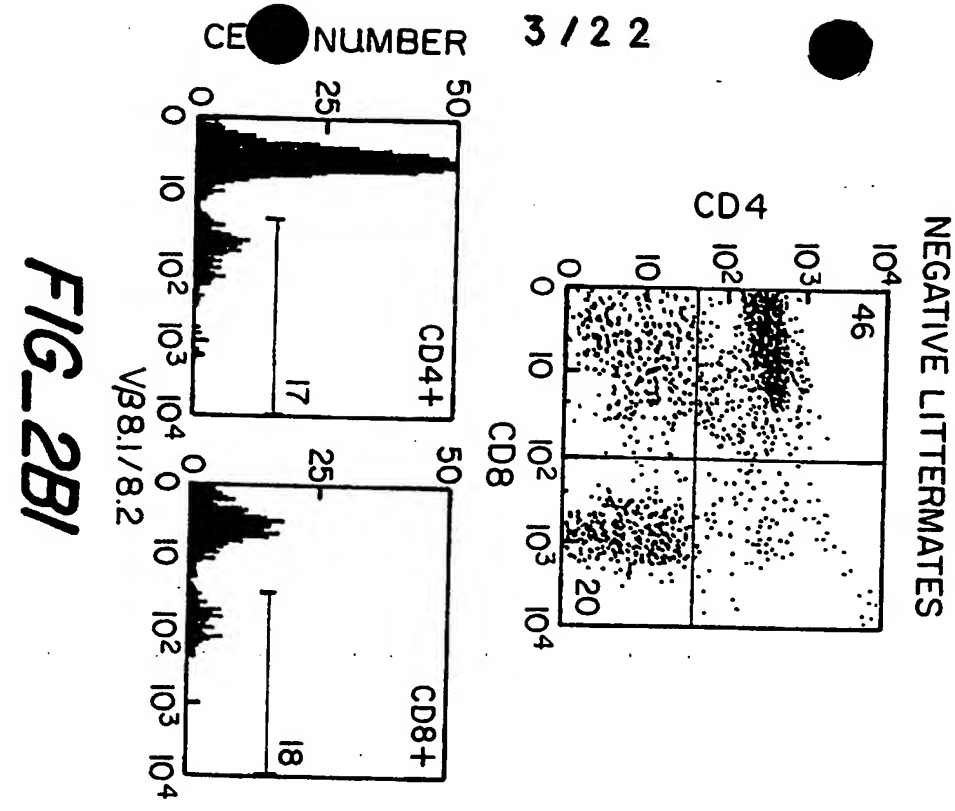

FIG_2B1

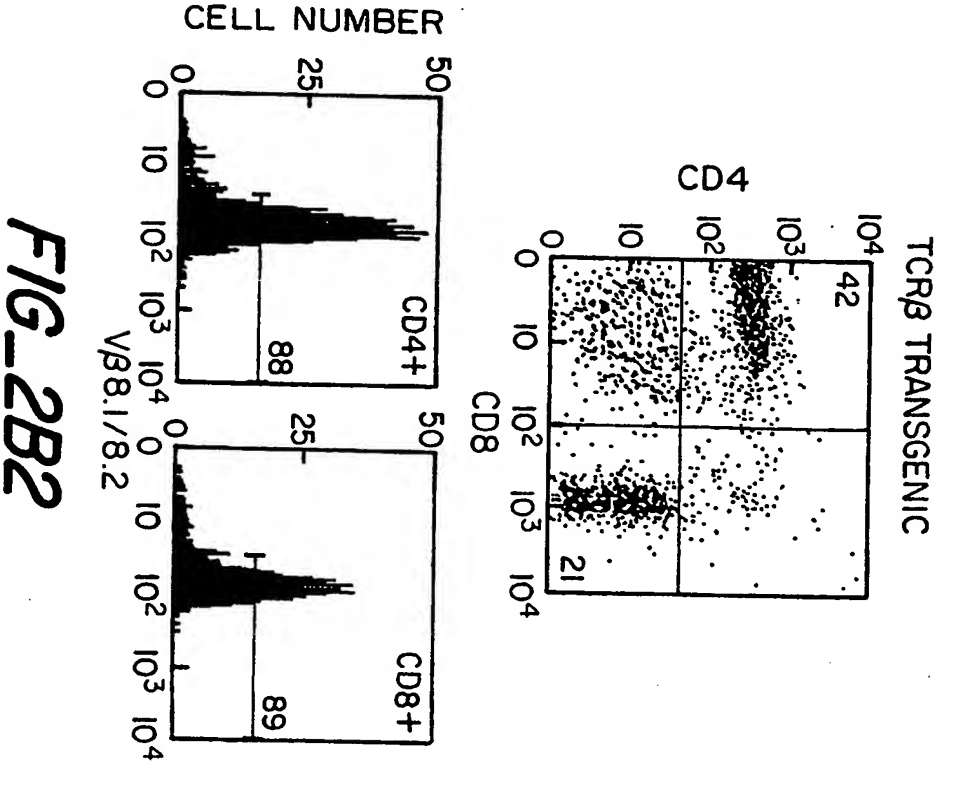

FIG_2B2

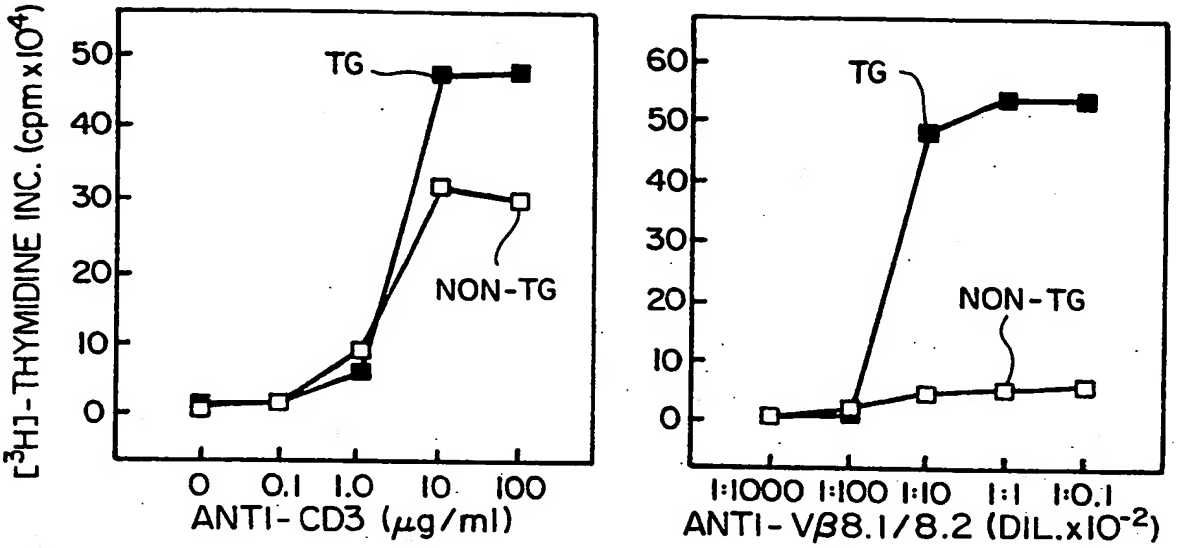
[3H]-THYMIDINE INC. (cpm x10^4)
50
40
30
20
10
0
TG
NON-TG
0 0.1 1.0 10 100
ANTI-CD3 (μg/ml)
60
50
40
30
20
10
0
TG
NON-TG
1:1000 1:100 1:10 1:1 1:0.1
ANTI-Vβ8.1/8.2 (DIL.x10^-2)

FIG_3

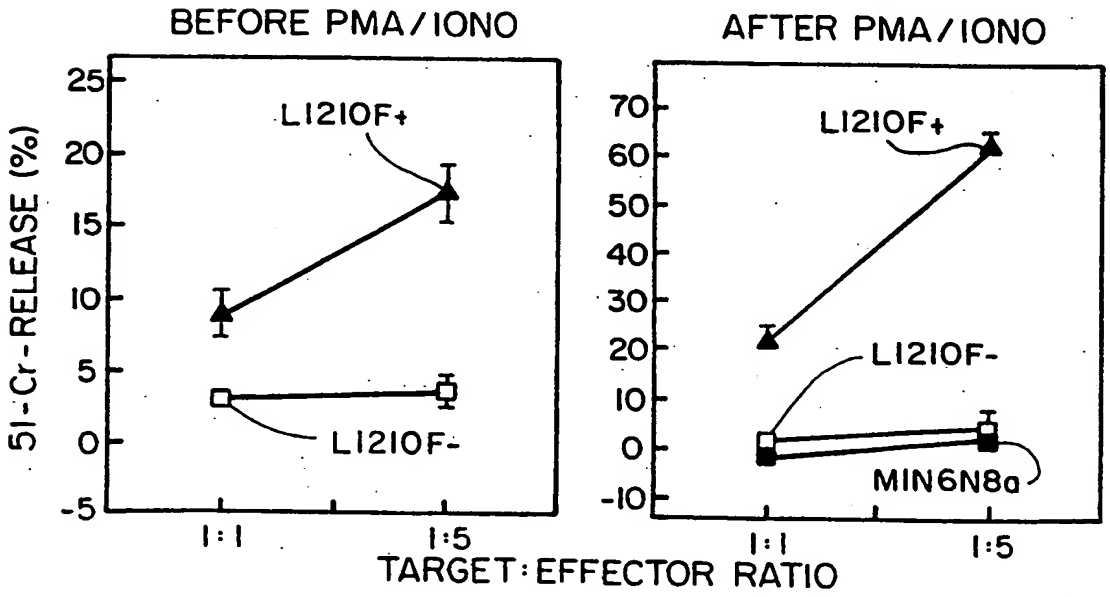
BEFORE PMA/IONO
AFTER PMA/IONO
51-Cr-RELEASE (%)
25
20
15
10
5
0
-5
L1210F+
L1210F-
70
60
50
40
30
20
10
0
-10
L1210F+
L1210F-
MIN6N8a
1:1
1:5
1:1
1:5
TARGET:EFFECTOR RATIO

FIG_4B

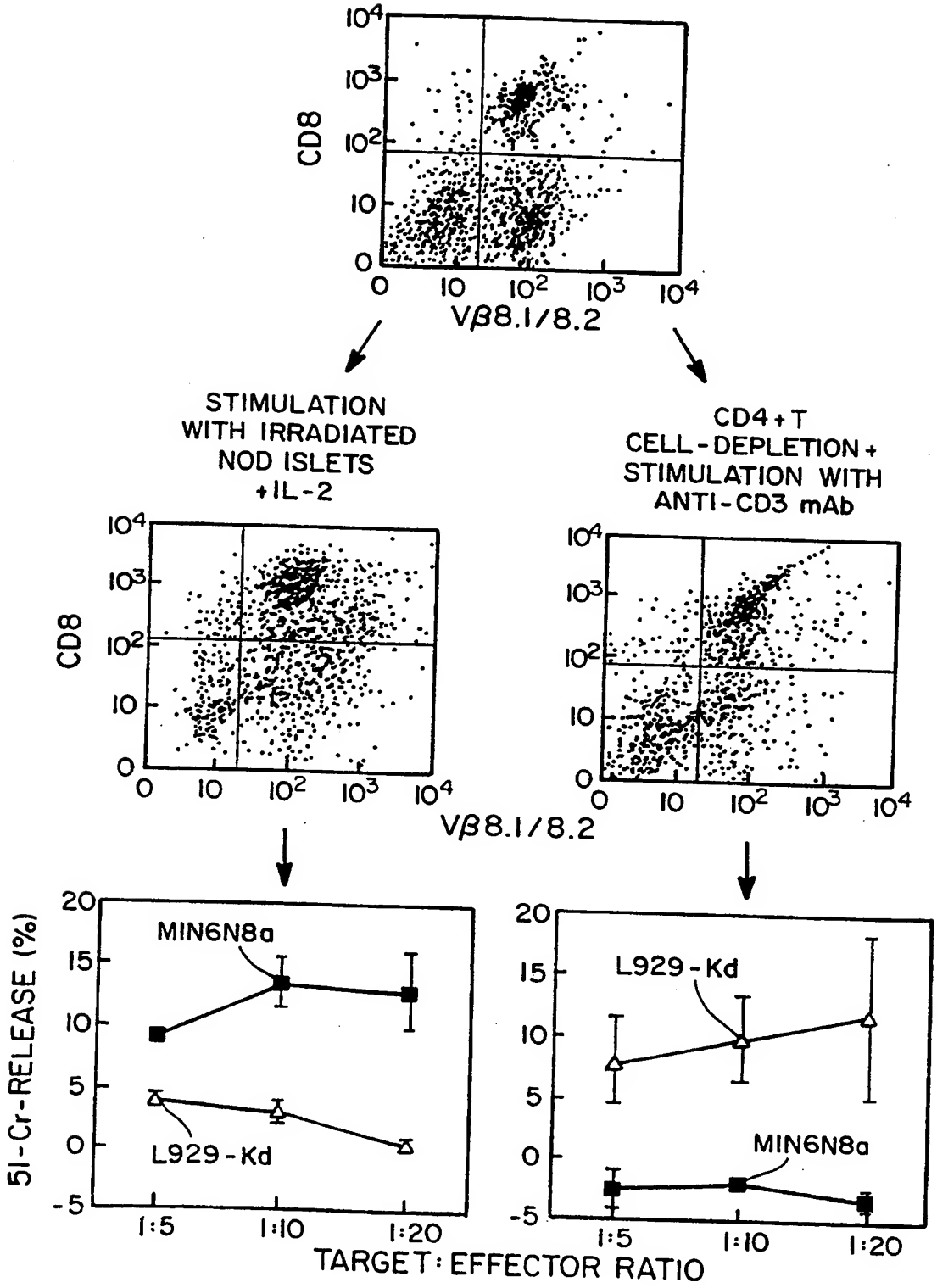
CD8
Vβ8.1/8.2
STIMULATION WITH IRRADIATED NOD ISLETS +IL-2
CD4+T CELL-DEPLETION+ STIMULATION WITH ANTI-CD3 mAb
51-Cr-RELEASE (%)
MIN6N8a
L929-Kd
TARGET:EFFECTOR RATIO
1:5
1:10
1:20

FIG_4A

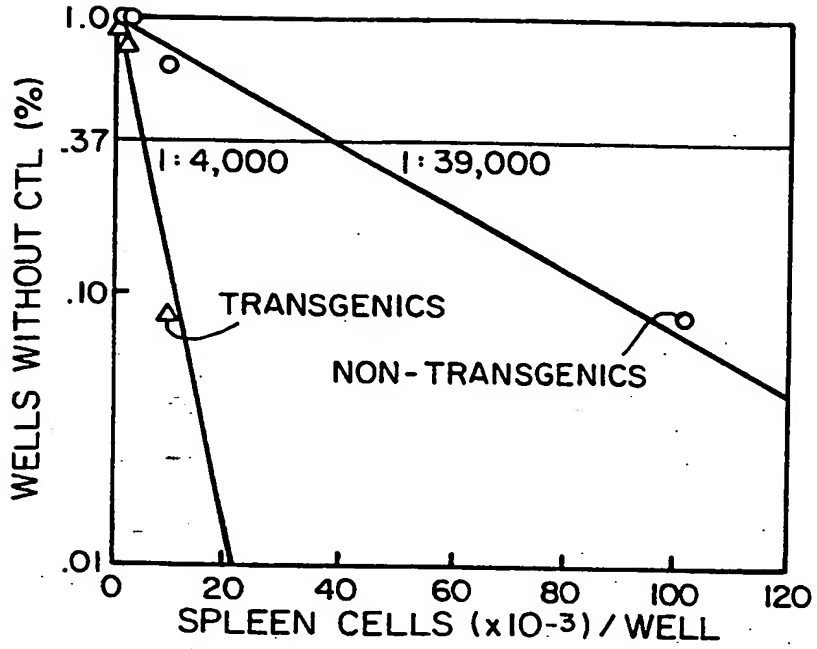
1.0
.37
.10
.01
WELLS WITHOUT CTL (%)
1:4,000
1:39,000
TRANSGENICS
NON-TRANSGENICS
0
20
40
60
80
100
120
SPLEEN CELLS (x10-3) / WELL

FIG_5A

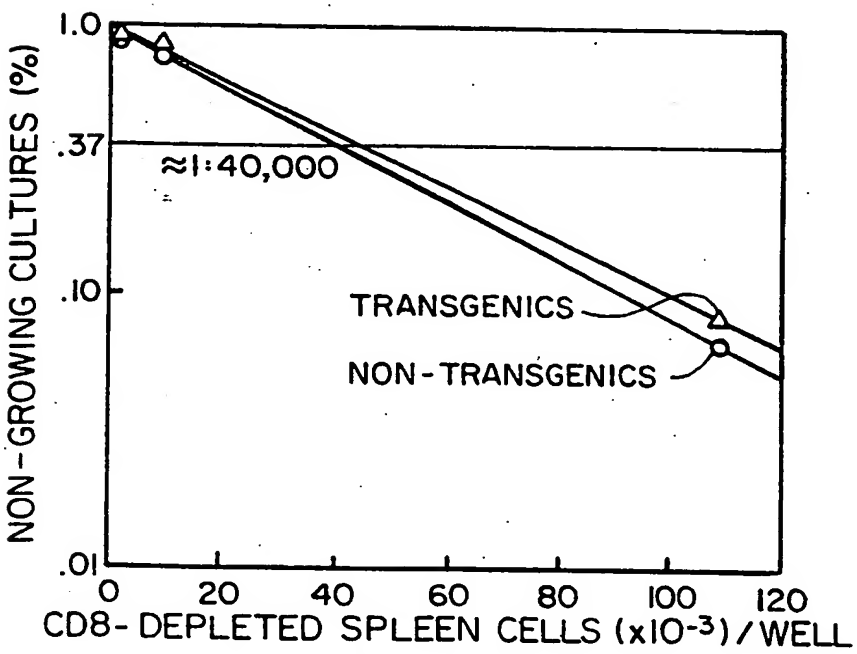
1.0
.37
.10
.01
NON-GROWING CULTURES (%)
≈1:40,000
TRANSGENICS
NON-TRANSGENICS
0
20
40
60
80
100
120
CD8-DEPLETED SPLEEN CELLS (x10-3) / WELL

FIG_5B

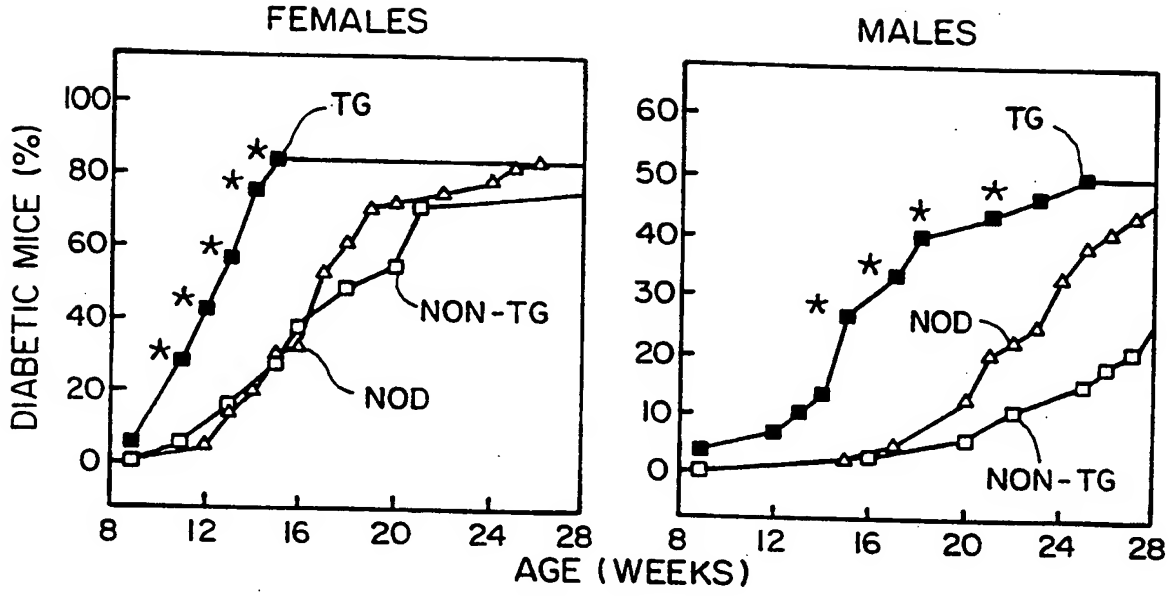

FIG_6

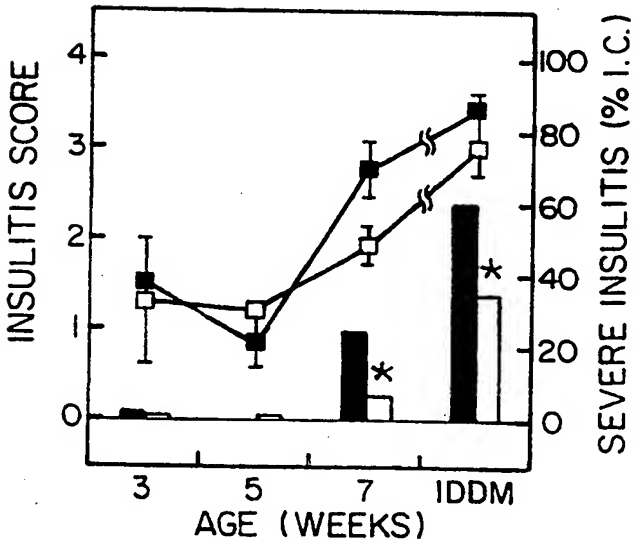

FIG_7A

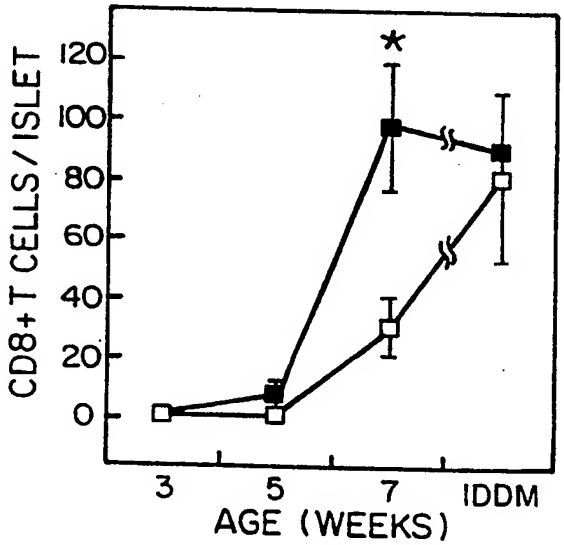

FIG_7B

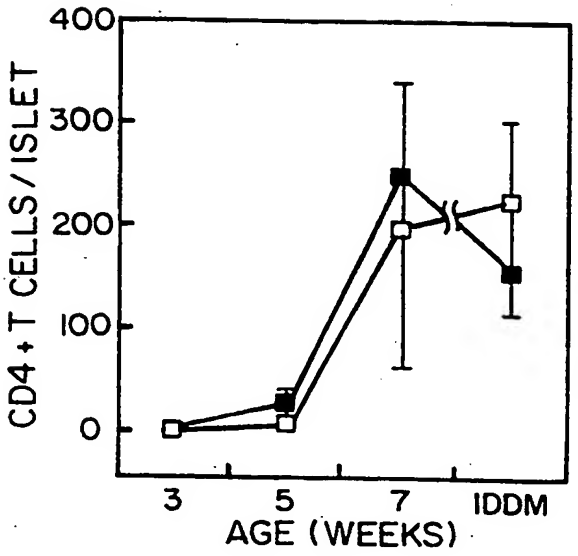

FIG_7C

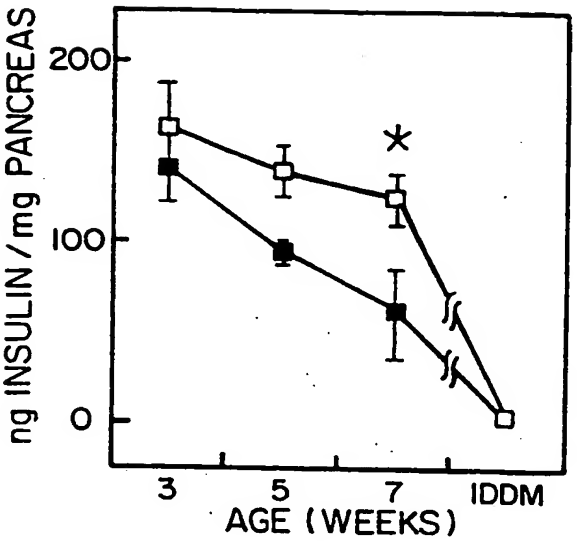

FIG_7D

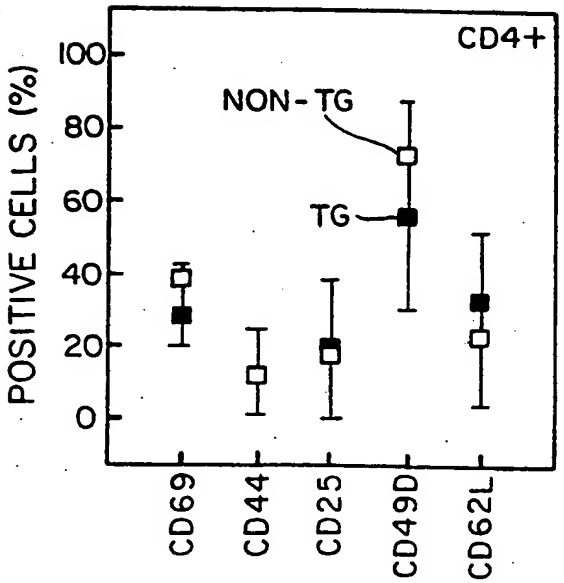

FIG_7E

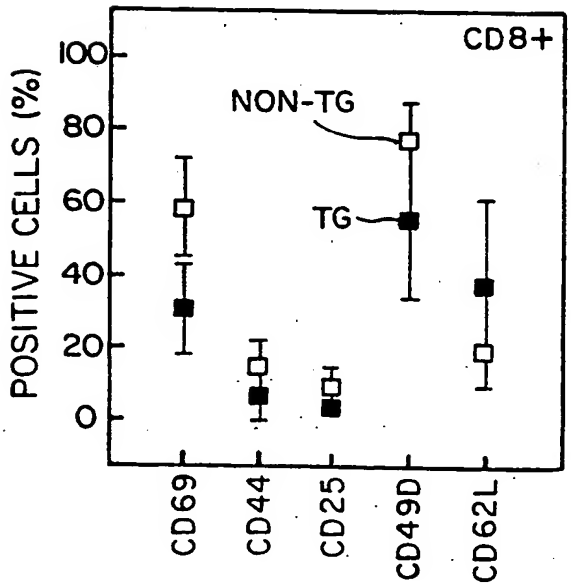

FIG_7F

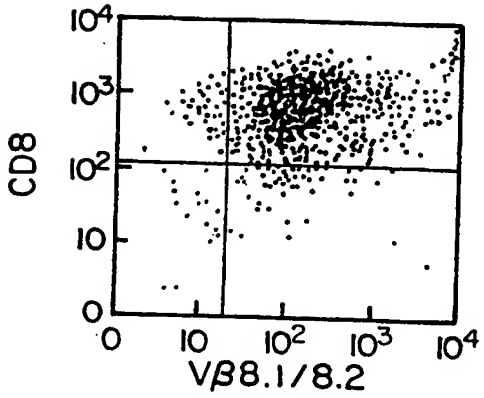

FIG_8A

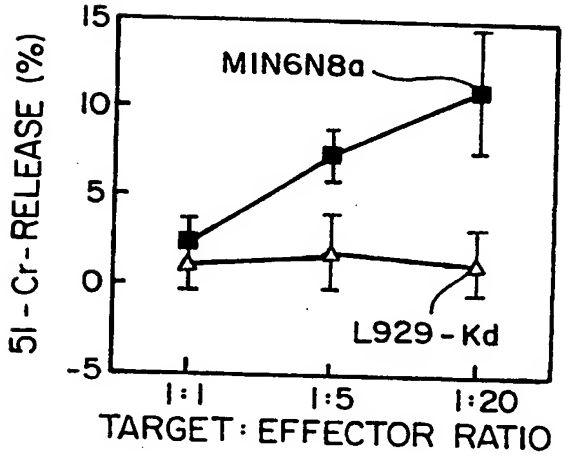

FIG_8B

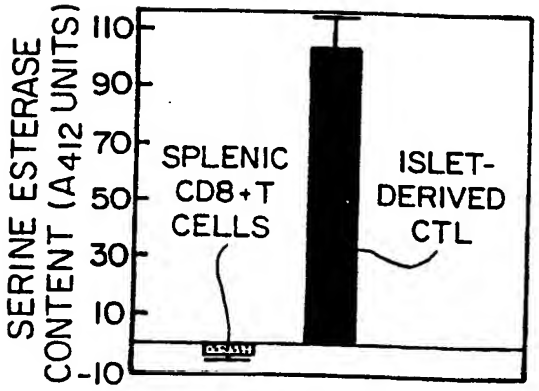

FIG_8C

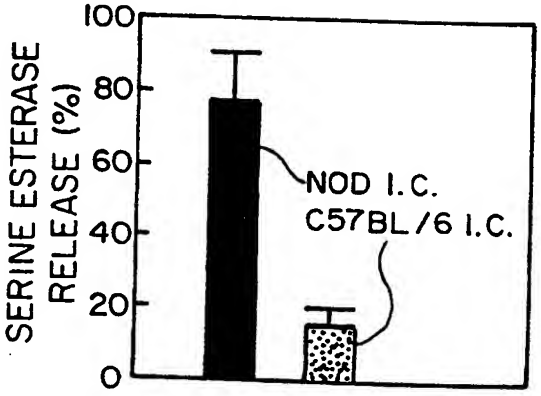

FIG_8D

DONOR CTL	Vα-Jα	N*	Vα	Nα CDR3		Jα
NY8.3	Vanl.1-Jα34	-	CA	MRD	S	GGSNAKLTFGKGTKLSVKSN
			TGT GCT	ATG AGA GAC	TCT	
CTL LINES						
272	Vanl.1-Jα34	16/16	CA	MRD	S	GGSNAKLTFGKGTKLSVKSN
			TGT GCT	ATG AGA GAT	TCT	
PI	Vanl.1-Jα34	13/18	CA	MRD	S	GGSNAKLTFGKGTKLSVKSN
			TGT GCT	ATG AGA GAT	TCT	
	Vanl.1-Jα34	3/18	CA	MRD	S	GGSNAKLTFGKGTKLSVKSN
			TGT GCT	ATG AGG GAT	TCT	
	Vα10-Jα48n	1/18	CA	LY		QGGSRKLIFGEGTKLTVSSY
			TGT GCT	CTC TAT		
	Vα1-Jα11	1/18	CA	ASS	NS	GTYQRFGTGTKLQVVPN
			TGT GCA GCT	AGC TCA	AAT TCT	
P3	Vanl.1-Jα34	10/16	CA	MRD	S	GGSNAKLTFGKGTKLSVKSN
			TGT GCT	ATG AGA GAC	TCT	
	Vanl.1-Jα34	2/16	CA	MRD	S	GGSNAKLTFGKGTKLSVKSN
			TGT GCT	ATG AGA GAT	TCT	
	Vα1-Jα11n	1/16	CA	ASE	S	NSGTSQRFGTGTKLQVVPN
			TGT GCA GCT	AGT GAG	TCA	
	Vα3-Jα34	1/16	CA	VSG	S	SGGSNAKLTFGKGTKLSVKSN
			TGT GCT	GTG AGC GGA	TCT	
	Vα8-Jα8	1/16	CA	STVG		NMGYKLTFGTGTSLLVDPN
			TGT GCT	TCC ACG GTC GGG		
	VanP3-Jα24	1/16	CA	PGN		NRIFFGDGTQLVVKPN
			TGT GCT	CCC GGC ACC		
P6	Vanl.1-Jα34	8/8	CA	MRD	S	GGSNAKLTFGKGTKLSVKSN
			TGT GCT	ATG AGA GAT	TCT	
2910	Vα4-Jα39n	14/14	CA	LRRMD	Y	ANKMIFGLGTILRVRPH
			TGT GCT	CTG AGG CGA ATG GAC	TAT	

FIG_9A

Vα - Jα	N*	Vα	Nα	Jα
Vαx - Jα15	2/17	CV	VA	RDGSALGRLHFGAGTQLIVI
Vα2 - Jα39	1/17	CA	ARGVD	YANKMIFGNGTIWRVRPH
Vα10 - Jα44	1/17	CA	MRA	GANTGKLTFGHGTILRVH
Vα3 - Jα19	1/17	CA	TVIDK	LASLGKLGFGTGTQVVVT
Vα8 - Jα4x	1/17	CA	WN	LSGSFNKWTFGAGNRLAVC
Vα1 - Jα34	1/17	CA		SGGSNAKLTFGKGTKLSVK
Vα4 - Jα18	1/17	CA	TEGP	LIFGQGTKLSIKP
Vα3 - Jα15	1/17	CA	VRG	GSALGRLHFGAGTQLIVI
Vα2 - Jα23	1/17	CA	AAYD	TNAYKVIFGKGTHLHVL
Vαx - Jα45	1/17	CA	AS	SGGSNYKLTFGKGTLLTVT
Vα5 - Jα14	1/17	CA	VSG	NSAGNKLTFGIGTRVLVR
Vα3 - Jα18	1/17	CA	TE	GKLIFGGGTKLSIK
Vα7 - Jα34	1/17	CA	S	SGGSNAKLTFGKGTKLSVK
Vα8 - Jα24	1/17	CA	L	NNNNRIFFGDGTQLVVK
Vα5 - Jα17	1/17	CA	VR	SSGSWQLIFGSGTQLTVM
Vα1 - Jα45	1/17	CA	V	NSGGSNYKLTFGKGTLLTVT

FIG_9B

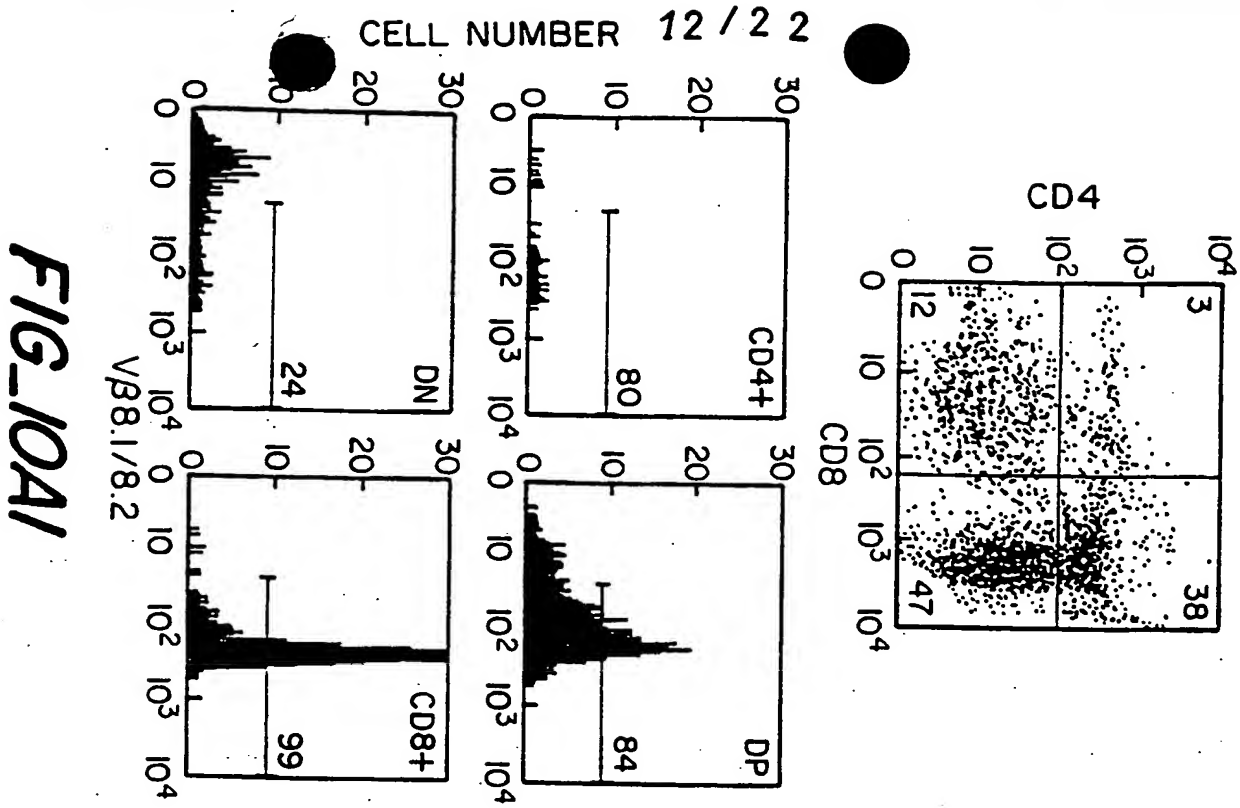

FIG_10A1

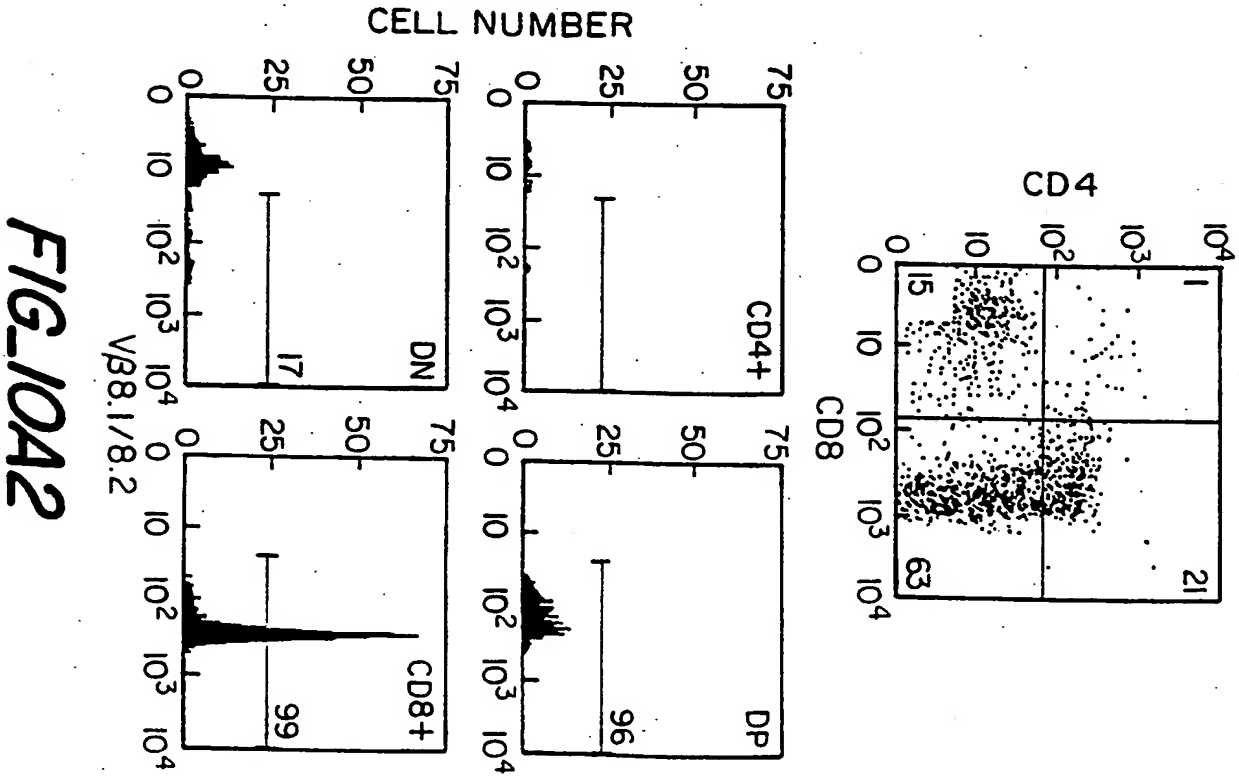

FIG_10A2

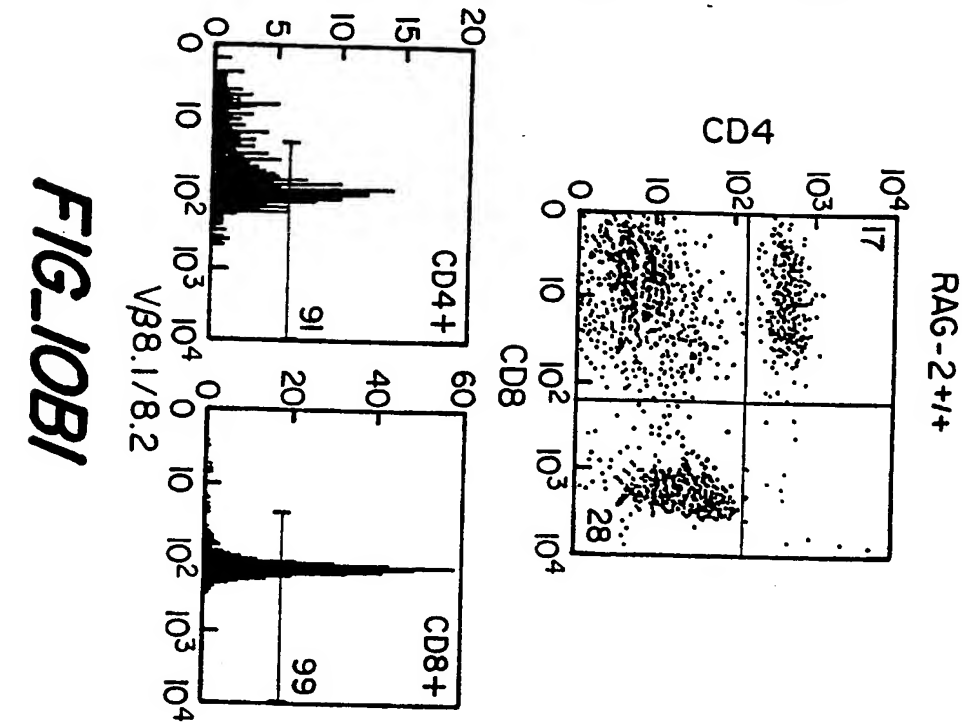

FIG_10B1

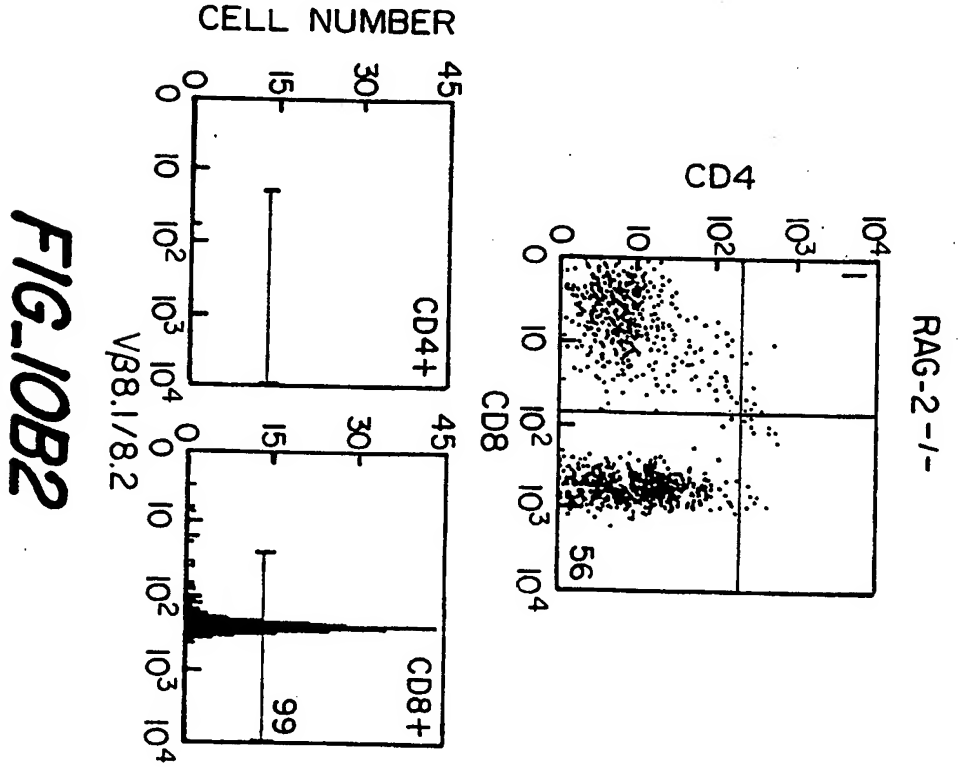

FIG_10B2

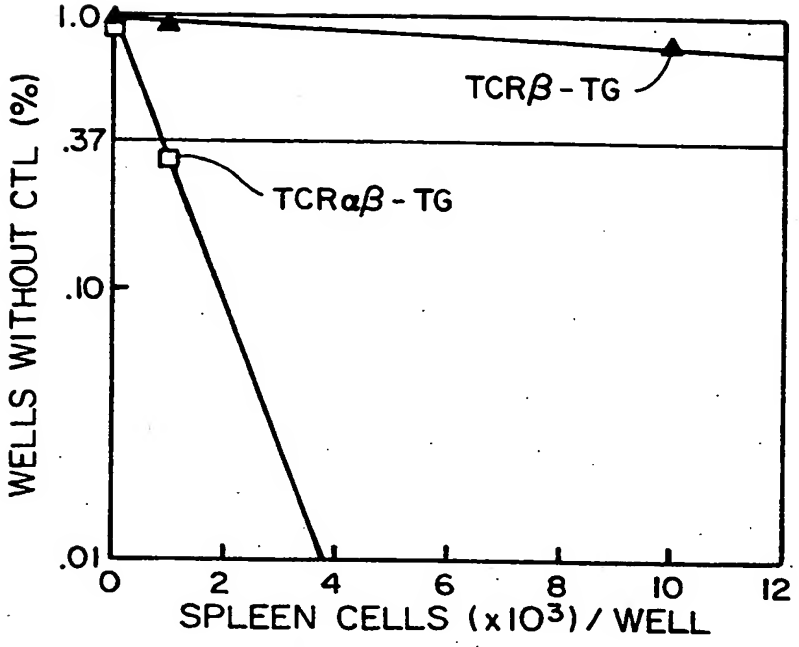
1.0
.37
.10
.01
WELLS WITHOUT CTL (%)
TCRβ-TG
TCRαβ-TG
0
2
4
6
8
10
12
SPLEEN CELLS (x10³) / WELL

FIG_11

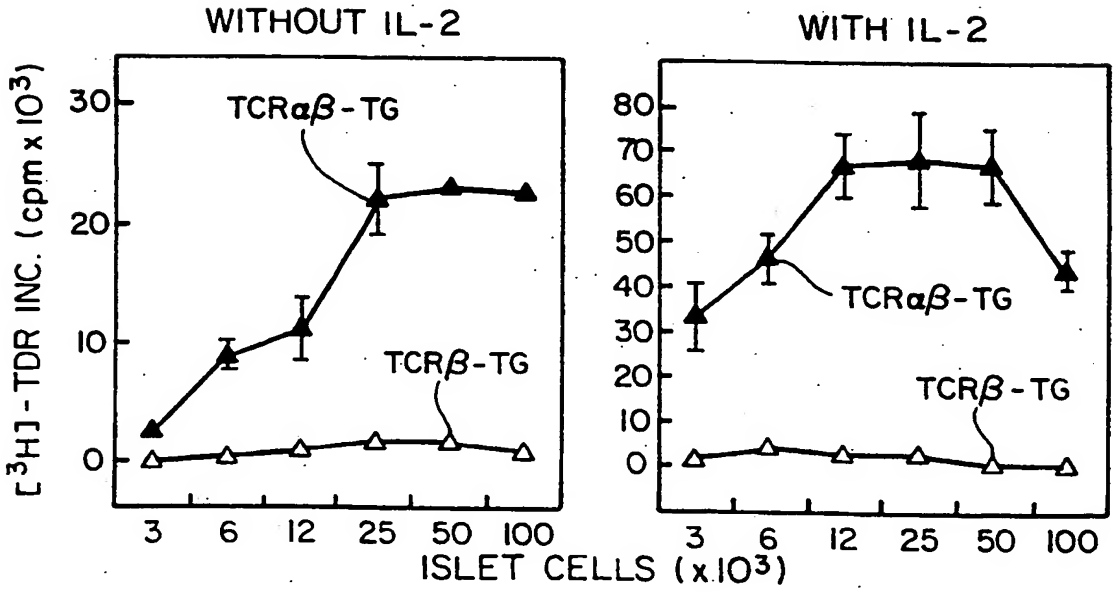
WITHOUT IL-2
WITH IL-2
[³H]-TDR INC. (cpm x 10³)
30
20
10
0
80
70
60
50
40
30
20
10
0
TCRαβ-TG
TCRβ-TG
3
6
12
25
50
100
ISLET CELLS (x10³)

FIG_12

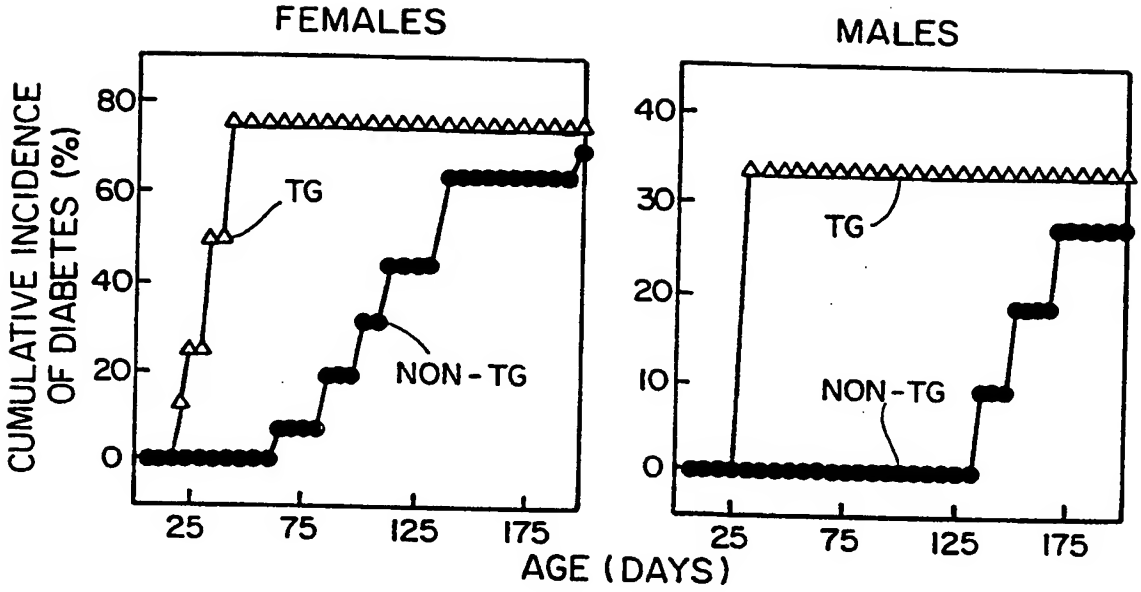
FEMALES
MALES
CUMULATIVE INCIDENCE OF DIABETES (%)
TG
NON-TG
AGE (DAYS)

FIG_13

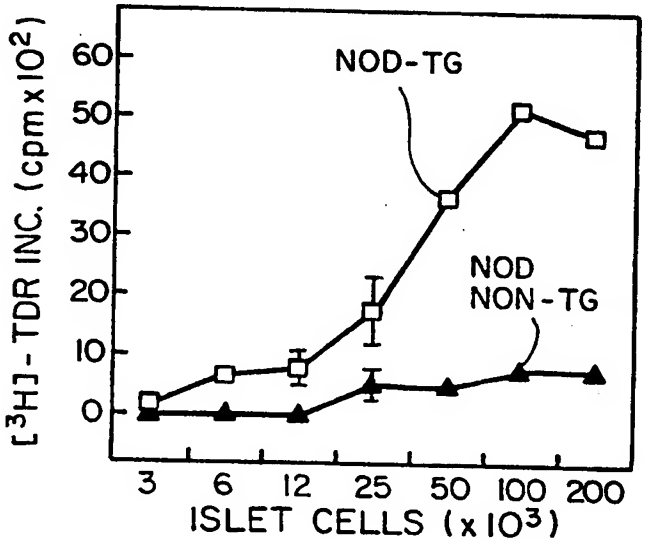
NOD-TG
NOD NON-TG
[3H]-TDR INC. (cpm x 10²)
ISLET CELLS (x 10³)

FIG_16

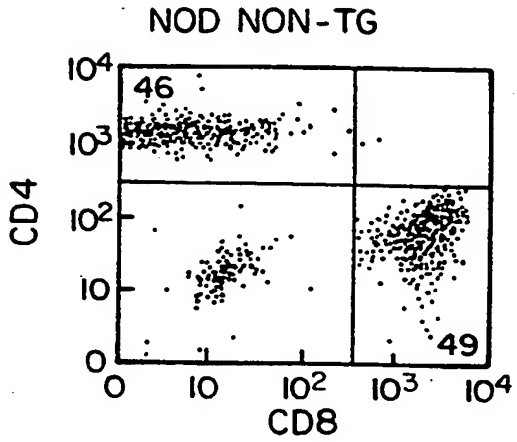

FIG_14A

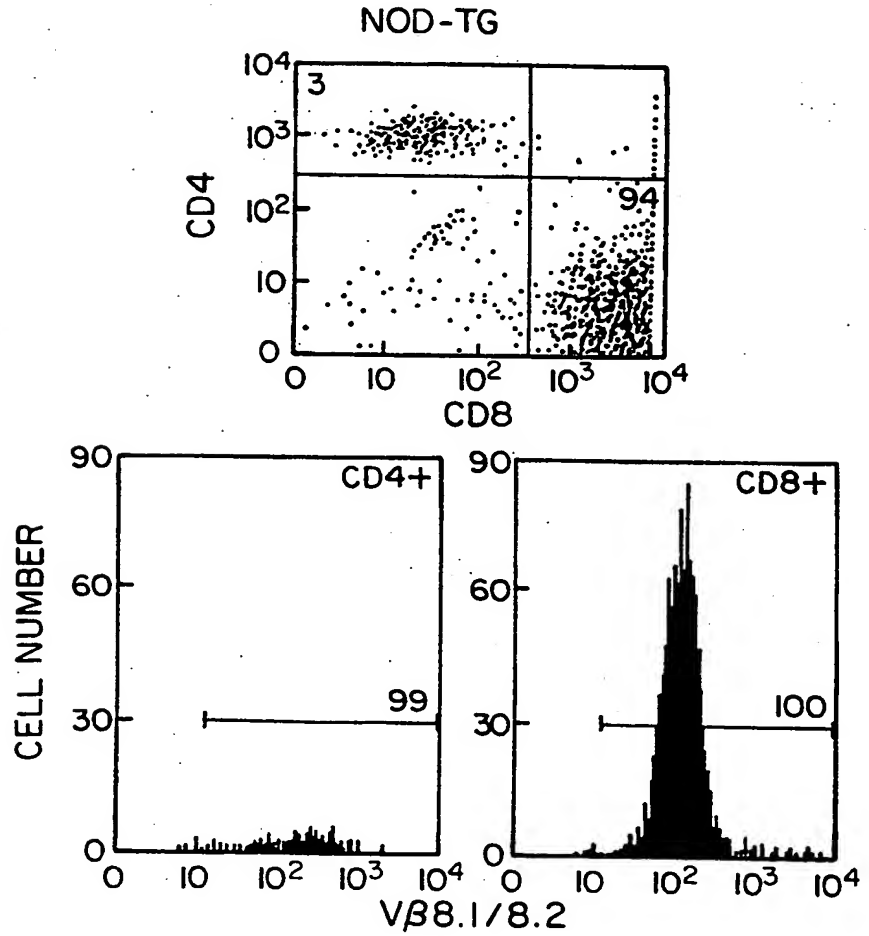

FIG_14B

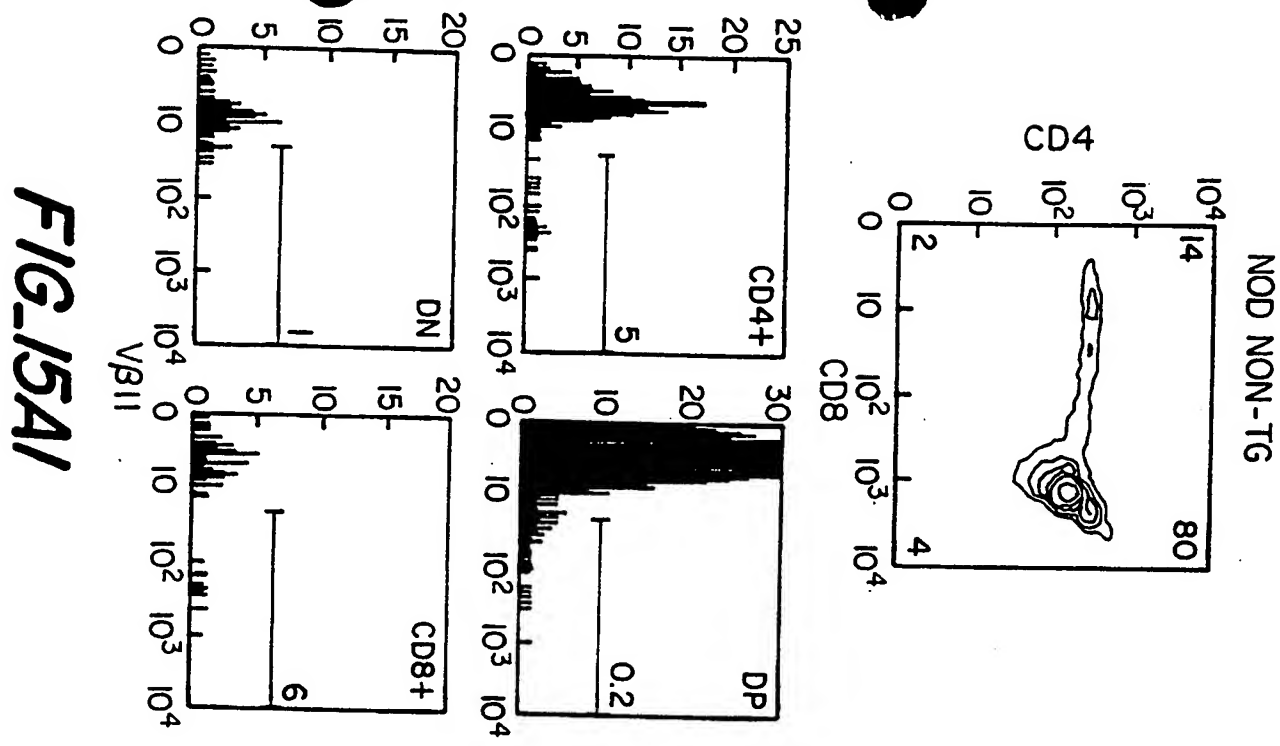

FIG_15AI

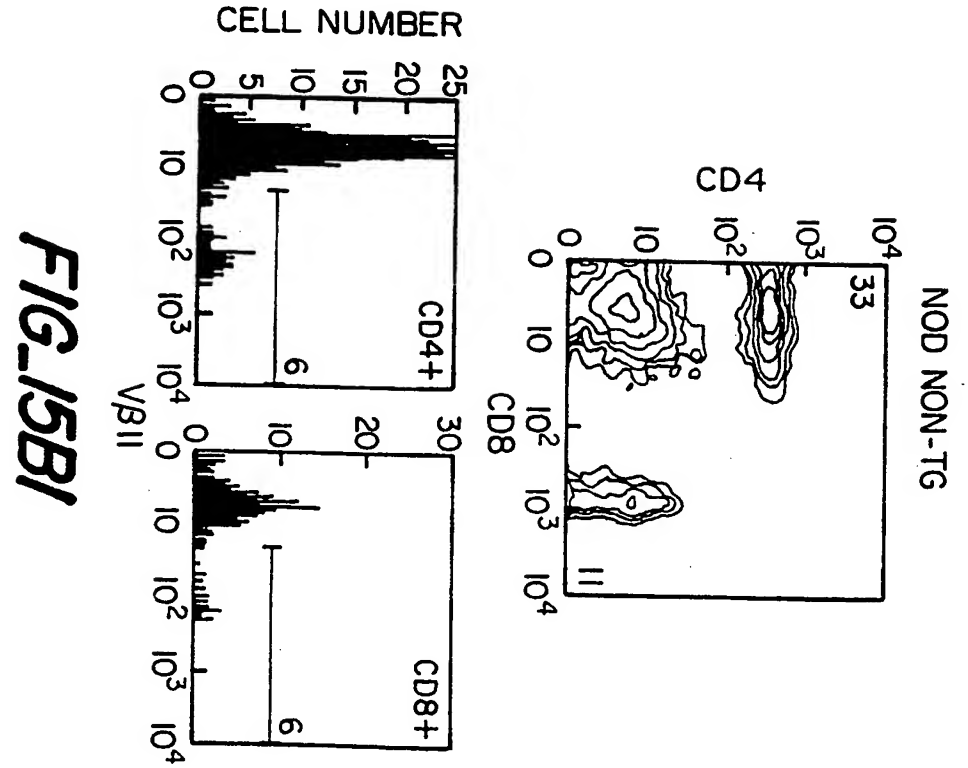

FIG_15BI

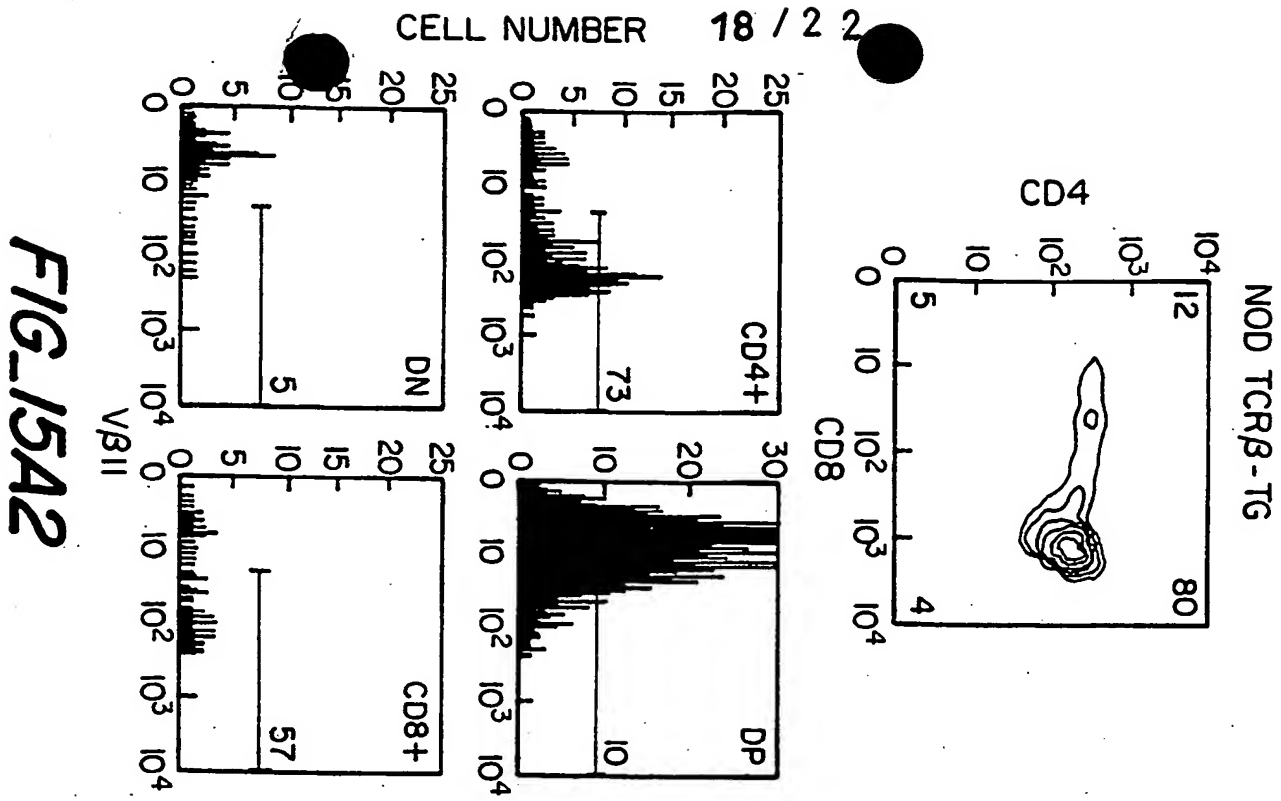

FIG_15A2

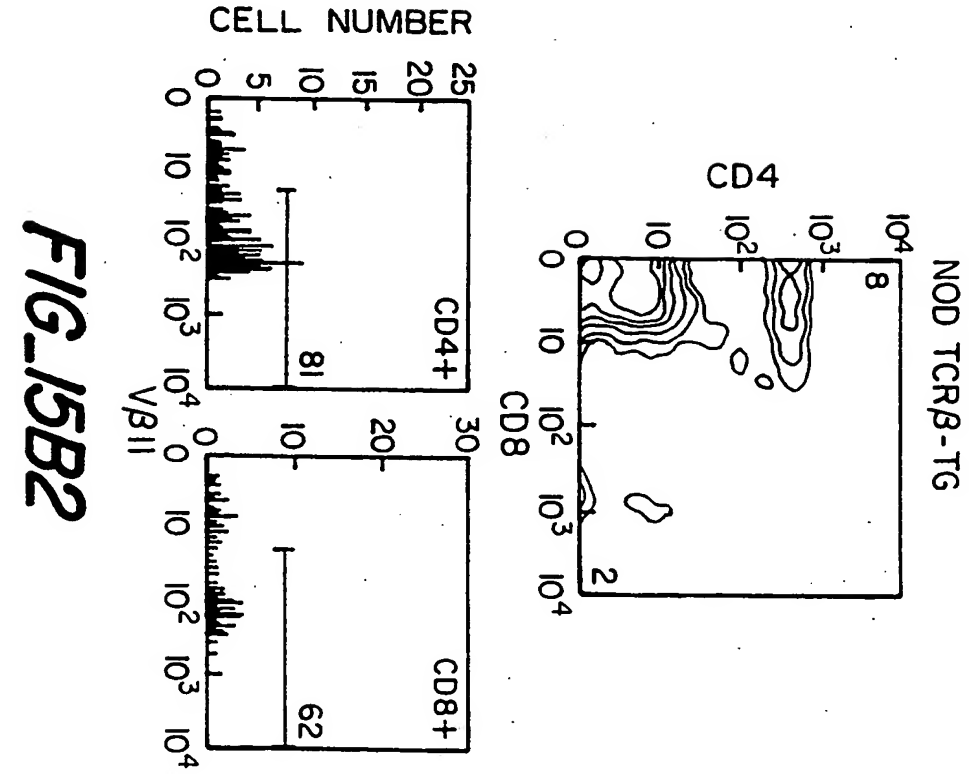

FIG_15B2

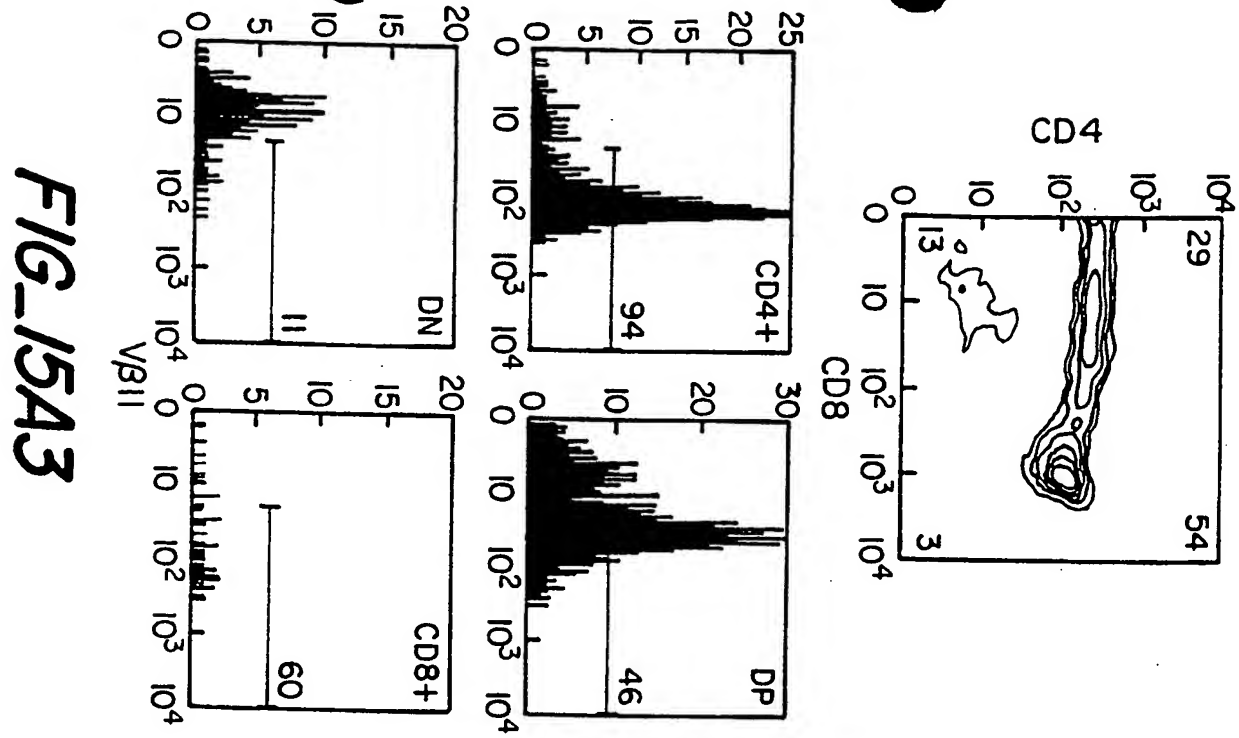

FIG_15A3

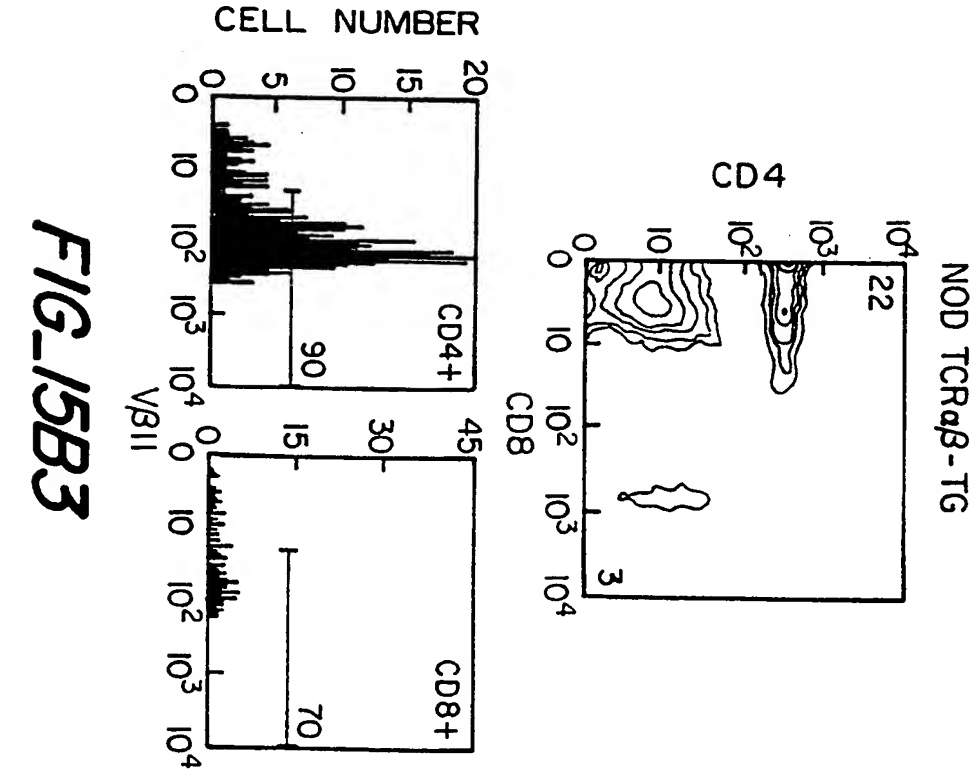

FIG_15B3

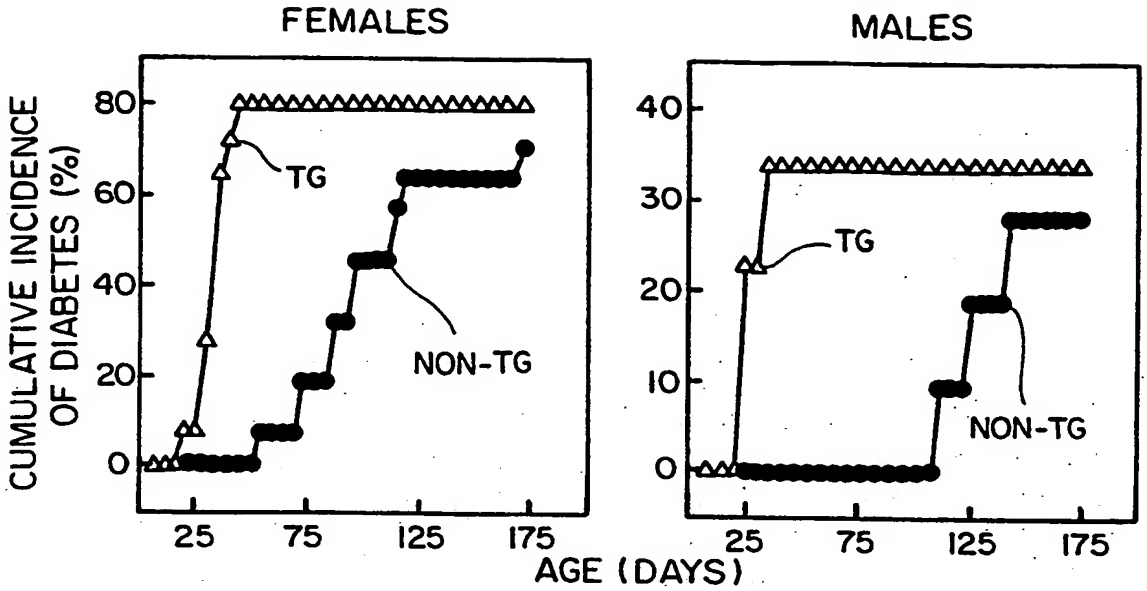
FEMALES
MALES
CUMULATIVE INCIDENCE OF DIABETES (%)
80
60
40
20
0
40
30
20
10
0
TG
NON-TG
TG
NON-TG
25
75
125
175
25
75
125
175
AGE (DAYS)

FIG_17

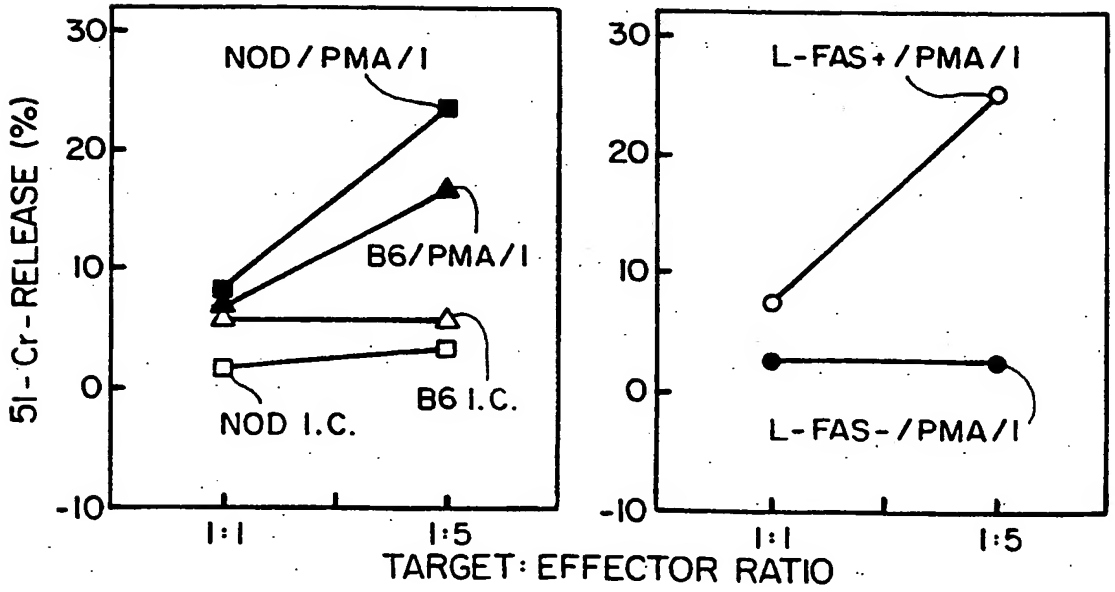
51-Cr-RELEASE (%)
30
20
10
0
-10
NOD / PMA / I
B6/PMA/I
B6 I.C.
NOD I.C.
L-FAS+ / PMA / I
L-FAS- / PMA / I
1:1
1:5
1:1
1:5
TARGET: EFFECTOR RATIO

FIG_20

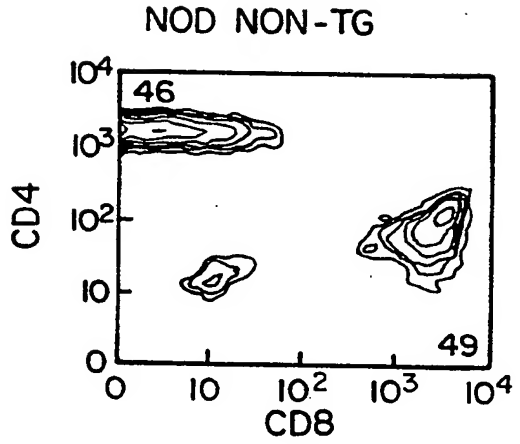

FIG_18A

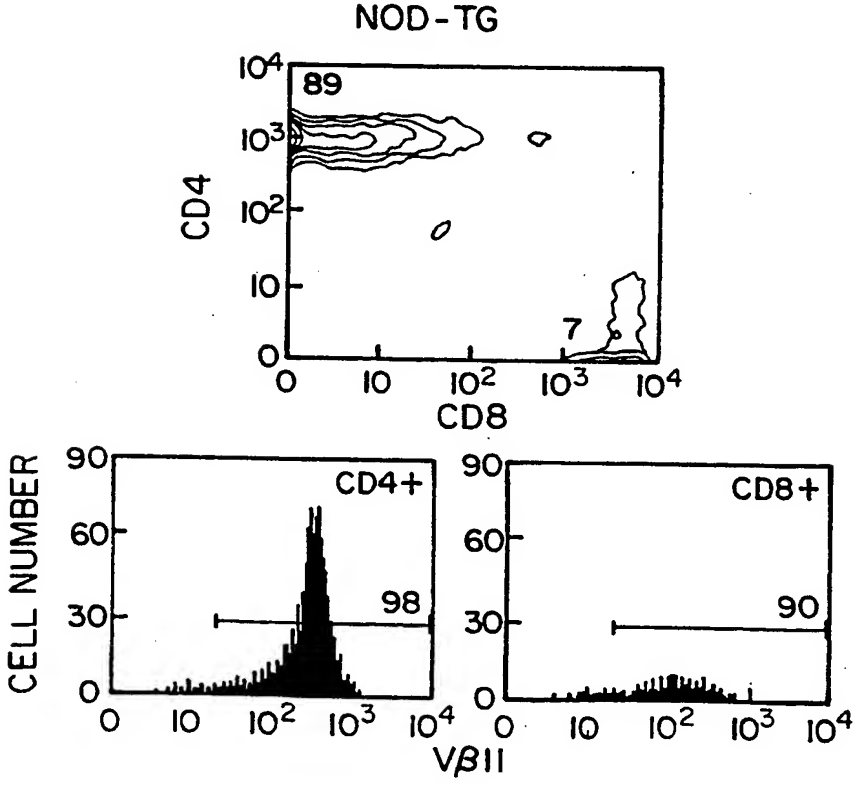

FIG_18B

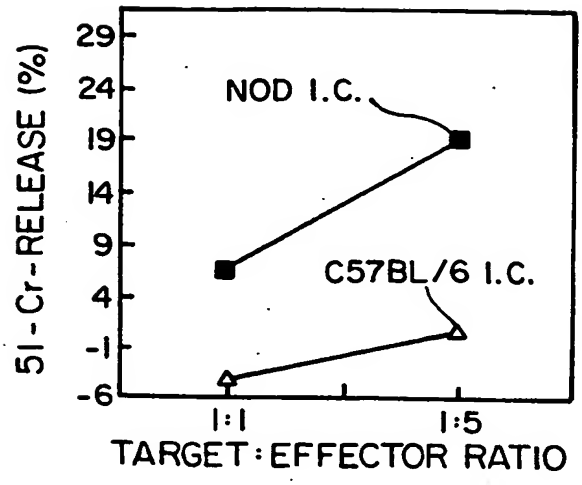

FIG_19A1

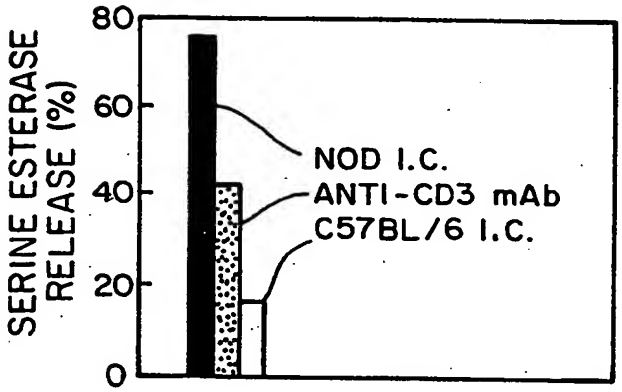

FIG_19A2

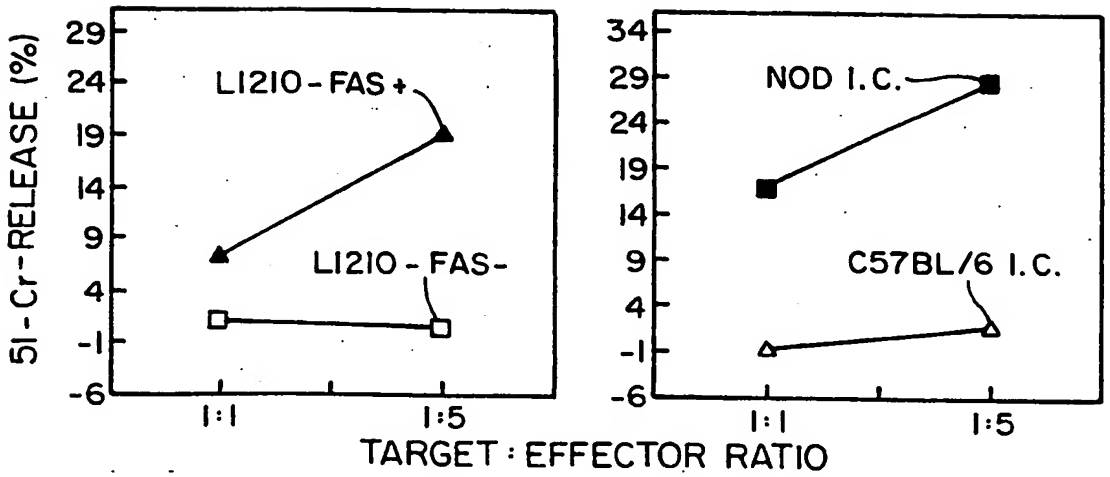

FIG_19B

INTERNATIONAL SEARCH REPORT

Inter[illegible]nal Application No
PC[illegible]/CA 96/00581

A. CLASSIFICATION OF SUBJ[illegible]MATTER
IPC 6 C12N15/00 [illegible]01K67/027 C07K14/725

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,91 19816 (UNIV LELAND STANFORD JUNIOR) 26 December 1991 see the whole document ---	1,8-11, 13-28
Y	JOURNAL OF IMMUNOLOGY, vol. 154, no. 5, 1 March 1995, BALTIMORE US, pages 2494-2503, XP000604922 SANTAMARIA, P. ET AL.: "Beta-cell-cytotoxic CD8+ T cells from nonobese diabetic mice use highly homologous T cell receptor alpha-chain CDR3 sequences" see the whole document --- -/--	1-6, 8-11, 13-28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 October 1996	25. 10. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Chambonnet, F

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Inte tional Application No
PC1/CA 96/00581

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of doc[redacted] with indication, where appropriate, of the relevant passages [redacted]	Relevant to claim No.
Y	WO,A,94 23760 (US) 27 October 1994 see claims	1-6, 8-11, 13-28
Y	WO,A,95 21623 (UNIV VERMONT) 17 August 1995 see page 8, line 12 - page 14, line 2; claims	1,8-11, 13-22
Y,P	WO,A,95 32285 (INST NAT SANTE RECH MED ;CENTRE NAT RECH SCIENT (FR); UNIV PASTEUR) 30 November 1995 see page 1, line 1 - page 6, line 10; claims 1,3,7,21,27,35 see page 11, line 8 - line 25	1-6, 8-11, 13-28
P,A	WO,A,96 21028 (PROCEPT INC) 11 July 1996 see claims 1,11,19,30,33-38	1,6, 22-28
A,P	EP,A,0 712 930 (SEO JEONGSUN) 22 May 1996 see claims	22-28

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 96/00581

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9119816	26-12-91	CA-A-	2086015	21-12-91
WO-A-9423760	27-10-94	AU-A-	6586494	08-11-94
WO-A-9521623	17-08-95	AU-A-	1912895	29-08-95
WO-A-9532285	30-11-95	NONE		
WO-A-9621028	11-07-96	NONE		
EP-A-0712930	22-05-96	NONE		